INHIBITION OF HERPES SIMPLEX VIRUS REPLICATION USING SMALL INTERFERING RNA THAT TARGET ICP4 GENE OF HERPES SIMPLEX TYPE 2

By

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This thesis is dedicated to my parents and to my husband

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Herpes simplex type 1(HSV 1) is the primary causative agent for ocular herpes and Herpes simplex virus type 2 (HSV 2) is the primary agent for genital herpes. Herpes simplex infections can be life-threatening in immunocompromised people and neonates. Herpes is a neurotropic virus that establishes latency causing a lifelong infection in sensory neuronal ganglia that supply the primary site of infection. Prophylactic oral antiviral therapy reduces recurrences of herpes simplex keratitis (HSK) by only 40% and has no sustained benefit following its discontinuation.[15] There are three kinetic groups of proteins that play a role in herpes simplex virus replication: immediate early, early, and late. Immediate early genes activate the host cell transcriptional machinery. There are five immediate early genes expressed in the earliest stages of the HSV infection cycle: α4 (ICP4), α0 (ICP0), α27 (ICP27/UL50), α22 (ICP22/ UL54), and α47 (ICP47/ US12). Small interfering RNAs (siRNA) are 21-23 nucleotide RNA molecules that cleave target mRNA sequences. In this study, we designed five siRNA molecules that target the

mRNA of the immediate early gene, ICP4. Two out of five siRNAs target both HSV 1 and HSV 2. All five siRNA molecules reduce HSV 2 (HG52) replication in rabbit skin cells, but there was no significant reduction shown in HSV 1 replication. The most effective siRNA molecule, siRNA19, was selected for the further studies that were carried out on the HSV 2 replication in different time points and in different multiplicities of infection. Treatment of RS cells with siRNA19 at 100 nM significantly reduced HSV 2 viral replication up to 92% at 12, 24 and 36, and 48 hours at low multiplicity of infection (MOI) of 0.0001, 0.001, and at a high MOI of 3 compared to transfection with an irrelevant siRNA. The siRNA molecules caused minor reduction in serum-stimulated proliferation of target cells, suggesting low off target effects. These results suggest that siRNA treatment targeting ICP4 may be an effective new therapy for treatment of HSV 2 genital herpes infection.

CHAPTER 1
INTRODUCTION

This thesis evaluates small interfering RNA (siRNA) as a possible therapeutic strategy in herpes simplex virus (HSV) infections. For this study, we mainly focused on designing and testing siRNA molecules that target the ICP4 gene of HSV 2. Furthermore, this study attempted to generate three siRNA molecules that would target the ICP4 gene of both HSV 1 and HSV 2.

Herpes simplex is a neurotropic virus that establishes latency causing a lifelong infection in sensory neuronal ganglia that supply the primary site of infection. HSV 1 is the primary causative agent of labial and ocular herpes, and HSV 2 is the primary causative agent of genital herpes. However, small percentages of genital herpes and facial herpes infections have been reported to be caused by HSV 1 and HSV 2 respectively. More than 50 million adults in the USA are known to harbor genital herpes which reflects an increased incidence of HSV infections in the country overall.[16] Herpes infection in neonates and in immunocompromised people could be life-threatening. Over the past forty years several successful antiviral agents have been used to treat herpes infections. Unfortunately, neither of these agents was successful as a cure for the disease. Lifelong antiviral treatment is indicated to have reduced the frequency and the duration of recurrences in immunocompromised people. However, it was reported that there has been no sustained effect after the discontinuation of prophylactic therapy. Over the next twenty-five years, it is estimated that the cumulative cost of incident HSV 2 infection in the United States will be forty-three billion dollars.[16] The unavailability of a cure for

herpes infections, the cost of treatments, and the emergence of resistance to existing antiviral therapies imply the importance of the development of a more effective treatment method for herpes infections.

Small interfering RNAs are double-stranded 21 nucleotide RNA molecules that are known to inhibit specific gene expressions at the post-transcriptional level. Previous studies have shown that siRNAs are effective in the inhibition of certain viruses such as HIV[6,7,8,30,33] Hepatitis C[38], Hepatitis B[23], and Human papilloma virus[24]. The exogenous delivery of siRNA offers a possibility of immediate delivery of therapeutic siRNAs for a short duration in acute infections, while short hairpin siRNA molecules expressed in plasmids or virus vectors exert a sustained effect that could be used in chronic infections.

The herpes simplex virus expresses three kinetic groups of proteins during virus replication, named immediate early, early and late proteins. ICP4 (α4) is an essential immediate early gene that is expressed at its peak at-4 hours post-infection. We hypothesized that the specific inhibition of ICP4 mRNA would also inhibit the HSV replication. Our overall goal was to design and test siRNA molecules that target the ICP4 gene of HSV 2. Our first specific aim was to identify the homologous sequences between ICP4 genes of HSV 1 and HSV2. It was expected that a site of a gene that shares a homology between two types of viruses has a better chance to be conserved in the longrun. Furthermore, such an approach would offer the possibility to design siRNA molecules that target both HSV 1 and HSV 2 infections at the same time. Our second specific aim was to design and test siRNA molecules in cell culture models. Our third specific aim was to evaluate the specificity of siRNA molecules on ICP4 gene silencing and also to evaluate the possibility of off-target effects on cell culture.

CHAPTER 2
LITERATURE REVIEW

Herpes Simplex Virus and Latency

Herpes Simplex Virus Infection, Structure and Virus Replication

Herpes viruses are enveloped, icosahedral, double-stranded viruses that belong to the herpesviridae family. All herpes viruses consist of an icosahedral nucleocapsid surrounded by tegument, which in turn is surrounded by an outer lipid membrane.[1] The tegument consists of proteins of which at least two proteins are believed to be involved in the transactivation of gene expression and one of the proteins shuts off host macromolecular synthesis. Eight glycoproteins in the virus envelope stimulate humoral immune response.[1]

Herpes viruses are categorized into three groups named alphaherpesvirus, betaherpesvirus and gammaherpesvirus. There are eight human herpes viruses that have been identified to cause diseases in humans: herpes simplex type 1 (HSV 1), herpes simplex type 2 (HSV 2), varicella-zoster virus (VZV), Epstein-barr virus (EBV), cytomegalovirus (CMV), human herpes virus type 6 (HHV 6), human herpes virus type 7 (HHV 7), and human herpes virus type 8 (HHV 8). Among them, HSV 1 and HSV 2 are known to cause facial and genital herpes infections. HSV 1 is the primary causative agent of facial herpes and HSV 2 is the primary causative agent of genital herpes. However, HSV 2 causes a small percentage of facial herpes and HSV 1 causes a small percentage of genital herpes.[46]

Herpes simplex type 1 and type 2 share almost all of the transcriptional regulations. Most of the features regarding HSV 1 replication have been identified. Both types are known to be able to replicate in a variety of cultured mammalian cells, animals and tissues. Thus *in-vitro* and *in-vivo* studies of these viruses have an advantage over other herpes viruses as they adapt well to a variety of animal models, and cell and tissue culture models.

The herpes virus has a complex genome that consists of a linear duplex molecule with two segments of unique sequences. These unique sequences, which are bounded by pairs of inverted repeats, are of 67 and 8.9 kb in length called unique long (U_L) and unique short (U_S) regions respectively.[1]Of the documented 80 viral proteins encoded in the HSV genome, 30 gene products are known to be responsible for the structural protein production of the virion.[46] Other than transcription of transcriptional and structural proteins, which are required for viral replication, the virus encodes for two important host modifying proteins, the transinducing factor (α-TIF/VP16/$V_{MW}65$ / U_L48/ virion stimulatory protein) and virion host shutoff protein (U_L41).[46]

The infection and virus replication begin by fusion of the virion envelop with the cell plasma membrane, and it takes approximately 18 hours to complete the lytic cycle. During this cycle, the viral DNA and one or more of the tegument proteins that are transported into the cell enter the nucleus. It is known that the cellular RNA polymerase II transcribes the virus DNA. The viral mRNA is then subjected to post-transcriptional modifications, such as cleavage, phosphorylation, sulfation, O- and N linked glycosylation, and myristilation.[1] A cascade of proteins synthesized during HSV

replication are grouped into three major kinetic classes; immediate early (IE or α), early (E or β) and late (L or γ).

The five immediate early genes named, α4 (ICP4), α0 (ICP0), α27 (ICP27/U_L50), α22 (ICP22/ U_L54) and α47 (ICP47/ U_S12), are expressed and peak at 2-4 hours post-infection. Since α4 and α27 map in the inverted repeat, they are diploid in the herpes genome. ICP4 has a high molecular mass of 170-175 kDA, which is known to be due to post-translational modification. ICP4 is known to be essential in viral replication, as it is needed for the auto regulation, and for the activation and transcription of the early and late genes. The active form of ICP4 protein is a homodimer and it binds to a specific sequence of α4 and α0 genes. (The binding sites in other genes are not yet certain.) The α0 protein is a diploid protein that is predicted to be approximately of 78 kDa, and known to have a synergistic interaction with α4 proteins. Although it is known that α0 is not required for the lytic infection of the cell culture, in low multiplicity, α0 is required for the significant expression of early and late kinetic proteins. The α27 protein is shown to be essential in viral growth as it acts as a repressor of α and some β genes and is also required for the expression of γ genes. It has been shown that α27 plays a role in the stabilization and export of improperly processed mRNA. There is minimal knowledge about the functions of α22 and α47. It is suggested that α22 plays a role in late gene expression while α47 has a supplementary effect. The α4 and α27 proteins have been shown to be targets for cytotoxic T lymphocytes; however, the role of α22 and α47 is not yet known.[1]

The seven of early (β) genes, DNA polymerase (U_L30), DNA binding protein (U_L42 and U_L29/ ICP8), ORI binding protein (U_L9), and the helicase /primase complex (U_L5, U_L8 and UL52) have been shown to be essential in virus replication, while the rest of the (β) gene products play only a partial role.[1] In addition to its DNA polymerase activity, the U_L30 acts as a DNA-dependent polynucleotide synthetase, 3'-5' exonuclease and RNase H. U_L42 contributes to the makeup of the DNA polymerase holoenzyme in a one-to-one complex. The complex formed by the three gene products of UL5, U_L8, and UL52 functions as a helicase and primase. The U_L29 or ICP4 encodes a major DNA binding protein that is involved in DNA replication and gene regulation. The essential (β) genes are mapped in the U_S region and the rest are mapped in the U_L region.[1]

The late or γ proteins are involved in the structure of the viral particle and are grouped into the two classes γ1 and γ2, in which the γ1 gene encodes for the capsid protein named VP5 while the γ2 gene encodes for the glycoprotein gC. The γ proteins consist of nucleocapsid proteins named VP5, VP19C, VP21, VP22, VP23, and VP24, and glycoproteins named gB, gC, gD, gE, gH, gI, and gJ. Among the glycoproteins, only gB, gD, and gH are known to be essential for virus multiplication in cell culture. Viral glycoproteins, especially gB or gC, are shown to play an essential role in the initiating event of the virus adsorption as it interacts with the heparin sulfate proteoglycans on the surface of the cell. The glycoproteins gB and gD are known to be involved in cell fusion and gH also is assumed to play a role in this virus entry process. The promoter sequences of two γ2 genes carry an α4 binding site. Thus, it is believed that α4 (ICP4) plays a role in γ gene expression. In addition, it has been shown that some other immediate early proteins such as α0, α22, and α27 and an early protein ICP8 play a regulatory role in

herpes virus γ genes. But α47 has not shown any effect on γ gene regulation. Since these are structural proteins, they may act as targets for the immune response. The α, β or γ gene transcriptional control can be considered a "nested set" situation since all three genes require the TATA box and both the α gene and the β gene require an additional distal signal and only the α gene requires an upstream TAATGART motif.[1]

Herpes Simplex Latency

Herpes simplex virus is an alpha virus, which is able to establish latency in the sensory ganglions of the nerves that innervate the primary infected site. The latency associated transcriptional unit (LAT) remains active during latency. It has been shown that infected mouse ganglia cells can either express LAT or HSV antigen, but not both. It was suggested that LAT could be down-regulating an acute transcription.[23] The Latency associated transcript is the only region of the herpes genome that is active during the latency of the virus. The LAT gives two stable transcripts of 2.3 and 1.8 kb. Since the last 750 bases of LAT are known to be complementary to the 3' terminus of ICP0 mRNA, it has been hypothesized that LAT may affect viral replication by limiting the expression of immediate early gene products. Furthermore, the 5' terminus of LAT was shown to facilitate HSV reactivation. It has been hypothesized that the LAT has a RNA specific ribozyme effect. This hypothesis was built up upon the facts that the both LAT transcript and ribozyme are restricted in the nucleus and also both do not encode any proteins. It was also found that the LAT shares other features with the ribozyme; for example, they both have a unique stable structure and both presence in lariat and nonlinear confirmation. Further, it was hypothesized that the LAT ribozyme inhibits the required synthesis factor for viral reactivation.[29] A study done in 1997 by Devi-Rao et al. on rabbit

eyes showed that a productive genome replication could occur in the corneas of reactivating rabbits with LAT+ or LAT – viral infections. But a greater number of viruses were observed with LAT+ infection compared to the LAT – infection.[10] Further, it was identified that two cAMP – response elements in latency associated transcripts were identified at –43 to –36 and the other at –85 to –78, and those elements were proposed to trigger reactivation via a second messenger pathway involving cAMP. It was suggested that this type of mechanism may be capable of translating stress signals from the cell to the latent viral genome.[4]

Although the cell culture is a useful system for studying the virus, it does not simulate the natural encounter of the virus, where the virus encounters different cell types. Thus some studies have developed a reporter virus to examine the cell tropism of Herpes simplex virus LAT expression in the sensory neurons. This system has enabled researchers to understand the mechanism of the establishment of the latent infection and the mechanism of reactivation. In such cases researches have observed a lower number of cells express LAT compared to the total number of infected cells.[3]

Herpes Simplex Virus Infection Exerts Immunity and Affects Cellular Protein Synthesis

The herpes simplex virus exerts both cell mediated and specific humoral immunities in the host. The damage such as keratitis is caused more by the cell mediated immune damage than by the infection itself.[13] Like other viruses, the herpes simplex virus infection affects cellular protein synthesis. It was shown a marked general decrease in transcript abundance during the virus infection with a few exceptions such as a limited number of stress response genes and cellular transcriptional regulatory genes. Increased expression of ISG 54K E2, JUNC, N-SHC, STAT5, and TAP1 transcripts can be given as

an example of a major effect on a limited number of stress response and regulatory genes. It was observed that the U_L54 ($\propto$27) protein plays a major role in the shutoff of the cellular proteins and is directly involved in a decline of host mRNA abundance. The US12 protein reduces the level of MHC (major histocompatibility complex) and also contributes to its primary function in the MHC class I pathway.[44] Further, it was known that the herpes simplex virus affects cellular transcription during its latency. Latency induced transcript and host functions are known to contribute to this repression. Some studies were carried out to determine the contributory factors that maintain HSV latency.[27] It was suggested that a local immune response might contribute to the maintenance of latent infection because of the presence of infiltrating immune cells and cytokines in the latently-infected ganglia. There is evidence suggesting that neuronal functions help maintain latency. Several genes in this category code for the neurotransmitter receptors, such as Gprc1g, Chrm1, and Gabbr, voltage-gated ion channels, such as Kcnc1 and Kcnab. There are proteins known to be involved in neurite extension, axonal elongation, and cell matrix interactions such as Prss 12, UIk1, COl15a1, Adam23, and n-chimerin, and proteins involved in catabolic functions, such as Man2b1, Ctsd, Usp20, Ube1x, and Acox2. All of these findings show that both viral and host cell functions contribute to the maintenance of latency.[27]

Treatment of Herpes Simplex Virus

There are several successful antiviral agents that have been developed over the past forty years. A number of factors such as the immune state of the individual, the site of infection, and the primary or secondary nature of the infection are considered when selecting treatment for herpes simplex virus infections. There are several diagnostic

methods to identify the HSV infection, but the isolation of herpes simplex virus in tissue culture is the most common and reliable. The antiviral agents, Acyclovir, Valacyclovir, or Famciclovir, are usually given to healthy individuals for 7-10 days during the primary infection. In current practice, the individuals who have frequent recurrences are treated with daily suppressive therapy. Other antiviral agents, including Trifluridine, Vidarabine, Foscarnet, Cidofovir and Docosanol are also used in herpes simplex virus treatment. Resiquimod is a topical immune modifier that is currently undergoing clinical trials and is known as an emerging therapy to delay recurrences.[36] Clinical studies have revealed an emergence of drug resistance in approximately 0.3% of immunocompetent hosts and 4 to 7% in immunocompromised patients with the use of Acyclovir, penciclovir and their pro-drugs for two decades. Furthermore, it was observed that the prevalence of Acyclovir-resistant HSV is higher in severely immunocompromised patients than in immunocompetent patients.[2,13,43] It was reported that Acyclovir resistance occurs mainly due to mutations in thymidine kinase mutations and less frequently due to mutations in viral DNA polymerase.[9,25,26,34] It was identified that these mutations resulted in decreased or absent HSV thymidine kinase production, altered the affinity of the thymidine kinase for acyclovir-triphosphate, or altered the affinity of the HSV DNA polymerase for acyclovir-triphosphate.[9,37]

Vaccine

Potential goals for HSV vaccination are prevention or decrease in the severity of the disease, protection of the sensory ganglia from latent infection, and prevention of recurrences in latently-infected individuals.[9]

Both cell mediated and humoral immune systems are known to act as protective immunities against HSV. It has been shown that immunization with DNA can induce

both humoral and cell mediated immunity whereas immunization with purified protein induces only a $CD4^+$ response but not $CD8^+$ or innate response.[32] Reinfection by HSV 2 in the genital tract is uncommon and this was suggested to be due to the cell mediated immunity by the primary infection. Furthermore, it was shown that antigen specific memory T cells in the genital tract provide a high level of protective immunity in the absence of a humoral immunity. The effectiveness of antibodies in protecting against neuronal spread is well-proven in animal studies. As for the prevention or the control of latency in cases of the herpes simplex virus, there have been several studies conducted to develop different vaccines, such as plasma DNA vaccines, vaccines with HSV glycoprotein subunits, replication deficient subunits vaccines, and recombinant virus vector vaccines.[32]

Herpes simplex virus type one is known to consist of an envelope that carries seven known antigenically distinct glycoproteins that are named gB, gC, gD, gE, gG, gI, gH, gJ, gK, gL, and gM. These glycoproteins are known to be inducers and targets for the humoral and cell mediated response of the host.[35] Herpes simplex virus glycoproteins are known to be the most favorable targets for a vaccine as they are highly immunogenic and induce neutralizing antibodies. Among them, glycoprotein B (gB) and/or glycoprotein D (gD) are preferred not only because they induce neutralizing antibodies, but also because they are the most abundant herpes simplex virus glycoproteins. Previously, it was suggested that the subunit vaccine using a mixture of recombinant glycoproteins is more efficient than a vaccine containing single recombinant alone.[36]

The DNA vaccines are typically composed of bacterially-derived plasmid DNA carrying eukaryotic gene regulatory elements that can express the specific antigens.

DNA vaccines are safer than the live attenuated vaccines, and are also capable of generating cell-mediated responses in the absence of any adjutants. Unlike subunit vaccines, DNA vaccines can be developed easily as multivalent vaccines because DNA vaccines can simply mix the plasmids that code for different antigens without affecting other components of multivalent vaccines. Further, studies done in animal models have shown that vaccines that have both proteins, glycoprotein B and glycoprotein D, provide better protection from the immune response than by vaccines that carry only one protein. Furthermore, it was shown that a plasmid coding for both gB2 and gD2 external domains as a chimeric protein has a better efficiency than a combination of both.[15] An effective herpes simplex virus vaccine would most likely need to elicit both cell-mediated and humoral immunity in order to achieve a high level of immunity.[32] Some studies have demonstrated that mice immunized with a DNA cocktail produce higher level of IL-12 and IFN-γ than mice immunized with a protein cocktail. Furthermore, it was observed that DNA vaccinated mice showed faster viral clearance, decreased intraocular viral titers, and increased IFN- γ and IL12 in comparison to the protein vaccinated mice group.[36]

Construction of a herpes simplex virus type 1 and type 2 bacterial artificial chromosomes (BAC) has been demonstrated. In addition to delivery of nonreplicating viral genomes, BAC could also be used to deliver the genome of a replicating virus for live virus immunization. However certain disadvantages, such as safety concerns and potential virus latency and persistence have hindered the development of a vaccine with BAC.[16] Immunity caused by purified protein vaccine is achieved by inducing antibody production and CD4+ T cell response but not CD8+ major histocompatibility complex

(MHC)-I- restricted CTL or innate response. In contrast, some other studies using HSV DNA encoding of the influenza virus nucleoprotein (NP) were shown to induce high titers of neutralizing antibody against the NP. Thus, immunization with DNA induces a more complete immune response compared to protein vaccines.[36]

It was demonstrated that intravaginal inoculation of HSV 2 could elicit immunity that can eliminate virus and provide a high level of protection against the spread of the virus via sensory ganglion. Some studies were carried out to find out the action of intranasal inoculation of attenuated HSV 2 in eliciting protecting immunity against genital herpes infection and spread via ganglion. It was demonstrated that comparable immunity could be elicited by the intranasal inoculation compared to the intravaginal inoculation and it was suggested that this immunity is mostly due to the humoral immunity.[32]

Herpes Simplex Virus and Gene Therapy

Herpes simplex virus has been successfully used as a vector in gene therapy.[21] They are large DNA viruses with the potential to accommodate large or multiple transgene cassettes. HSV 1 is preferred because it establishes latency in neurons. Latency is a state in which viral genome persists in the host as intranuclear episomal elements.

It has been demonstrated that herpes simplex virus growth can be inhibited by the Rnase P ribozyme.[45] Ribozymes are RNA enzymes that can specifically cleave a RNA sequence of choice. These demonstrate a catalytic and irreversible cleavage of the target RNA. It has been observed that both hammerhead and hairpin ribozymes could inhibit viral replication.[45]

Virus and RNA Interference

RNA Interference

Double-stranded RNA has been shown to silence gene expression in a wide variety of organisms.[14] This silencing can happen at post-transcriptional or transcriptional levels. Hence this mechanism is divided into two categories: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). The cells do not impede their own gene expression. Therefore an RNA trigger for gene silencing has to be recognized as foreign to the cells. The double-stranded nature of a silencing RNA is considered one potential character to be recognized as foreign to the cells. PTGS acts by decreasing the half-life of RNA. Therefore, the naturally stable RNAs are dramatically affected compared to the RNAs that are rapidly synthesized and degraded. It was hypothesized that a decrease in mRNA might activate the RNA production that restores the expression level. Several models were hypothesized showing how the RNA involved in PTGS can trigger chromosome modification.[19] The long double- stranded RNA molecules used for RNA interference haven't shown success in gene silencing in vertebrates because long-double stranded RNA activates the vertebrate interferon response.[12] It was shown that the interfering RNAs (RNAis) identified in plants are initially cleaved into small interfering RNAs (siRNAs), which are ~21 nt long double-stranded RNA (dsRNA) molecules having 2 nt 3' overhangs. This reaction is mediated by a cytoplasmic Rnase III family enzyme called dicer and these double-stranded RNA molecules are termed small interfering RNAs (siRNAs). One strand of this siRNA duplex is incorporated into the RNA induced silencing complex (RISC), a large protein complex. The RNA guides the complex to the homologous mRNA molecule and then cleaves the target mRNA. Finally, the RISC will be released and the cleaved RNA will be degraded. The synthetic siRNAs

have been discovered and shown to be effective in RNA silencing. A synthetic siRNA has only a 19 nt long double-stranded region, which won't activate the interferon system.[13]

Small interference RNAs (siRNAs), 21-23 nucleotide long double-stranded RNA molecules could specifically cleave targets mRNAs. The siRNA cleavage action is not affected by the secondary structure of mRNA and the efficiency of siRNA is determined by the siRNA specific properties. This property is in contrast with the antisense, where silencing is dependent on mRNA properties such as target site accessibility, which is determined by local mRNA confirmation. Some studies carried out by Elbashir et. al have come up with selection criteria for the design of siRNA sequences as follows. They have shown that the siRNA should be 21 nt long, and the sequence should preferably be 50 to 100 nt downstream of the start codone. The selected sequence site should not be in untranslated regions. The selected sequence should carry 5'-AA (N19) UU, where N is any nucleotide in the mRNA sequence. Further, they recommended performing a blast search of a respective organism to ensure a specificity of selected siRNA sequences on the cleavage of the target sequence.[18] Recent studies done by A Reynold et. al were able to publish eight characteristics associated with siRNA functionality, such as low G/C content, an absence of inverted repeats, proper internal stability at the sense strand 3'-terminus, and base preferences at positions 3, 10, 13 and 19 of the sense strand. It was shown that there was an improved efficiency of siRNA that were designed according to the above criteria.[39]

MicroRNAs (miRNA) are encoded within the eukaryotic genome as short hairpin RNA structures. It was proposed that a long hairpin structure (priMRNA) is cleaved by

an enzyme termed RNase III or Dorsha, into ~70 nt long intermediates termed pre-mRNAs. These pre-mRNAs are processed by the dicer into ~21 nt molecules termed miRNAs which specifically block the translation rather than degrading the mRNA. In contrast to siRNA molecules, microRNA molecules have one or two mismatches in the stem while siRNAs are perfectly double-stranded. It was shown that miRNA can function as siRNA to degrade mRNA when they encounter a perfect mRNA target. Similarly, siRNA was shown to function as miRNA, thus blocking the translation of target mRNA when there are few mismatches between the siRNA and the target mRNA.[14]

Synthetic siRNAs are expensive and transfection is not efficient in all cell types of interest. The effects of siRNA won't last long due to the degradation and dilution of siRNA in the subsequent cell replication. Several groups have developed short hairpin RNAs that are structurally analogous to pre-mRNA hairpin intermediates, using promoters that depend on the RNA polymerase III.[13]

In addition to the reported success of siRNAs in cancer research, plant and gene studies, siRNAs have also shown effectiveness in reducing the replication of certain viruses such as Hepatitis B, Hepatitis C, HIV, Human papilloma virus and Poliovirus.[5,7,8,20,24,31,38]

Off-Target Effect of siRNA

It was observed that siRNA exerts non-specific effects called off-target effects. These effects might be due to a siRNA effect or miRNA effect of the oligonucleotide molecules on the off-target mRNAs. However, some studies have shown that siRNA molecules don't activate the IFN response, which is known to be activated by long double-stranded RNA molecules in the cells.[40]

In contrast, some studies were able to demonstrate that siRNA-mediated gene silencing has sequence specificity and does not induce detectable secondary changes in the global gene expression pattern.[12]

Several possible nonspecific effects such as cross-hybridization and degradation of nonspecific mRNA, aptamer effects due to the binding with cellular proteins in a sequence specific manner, interference in translation by miRNA effect, and induction of dsRNA response were identified as potential ways to, exert nonspecific effects of siRNA. It was shown that siRNA is highly specific for targeted gene knockdown by microarray analysis.[41] In contrast, it was demonstrated that siRNA can exert its nonspecific effect by cross-hybridization.[41]

CHAPTER 3
MATERIALS AND METHODS

Cell Culture

The effect of siRNA on the replication of HSV 1 and HSV 2 was measured using cell cultures. Rabbit skin cells were grown in the minimum essential medium supplemented with 5% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were grown in the minimum essential medium supplemented with 10% fetal bovine serum 100 units/ml of penicillin, and 100 μg/ml of streptomycin. During transfections the cells were grown in the same type of medium without antibiotics. Cells were incubated in a 5% CO_2 humidified incubator at 37°C. Cell passages were carried out regularly and cells were not maintained beyond 30 passages after thawing the stock culture.

Assessment of transfection efficiency to determine the optimal siRNA concentration was done using Lab-Tek II Chamber slides. Confluent 250 ml (75 cm^2) flasks were trypsinized, cell suspensions were diluted 1:10, and 125 μl of aliquots were transferred into each chamber.

All the experiments were carried out in 35 mm cell culture dishes. One day before siRNA transfection, the confluent cells grown in 250 ml (75 cm^2) cell culture flasks were trypsinized with 5 ml of trypsin. After adding 10 ml of antibiotics free medium to the trypsinized sample the cells were centrifuged at 2000 × g for 5 minutes. The cell pellet was resuspended in 16 ml of antibiotics free medium. 500 μl of cell suspensions were

transferred into the dishes that contained 2.5 ml of antibiotic free medium. The experiments carried out to assay the plaque forming units in the same dishes were seeded with the cells in 1:14 dilution to achieve 100 % of confluency at the time of infection.

The plaque assays were carried out in 24 well plates. One day before the infection, 2 ml of medium were added to the dishes. Confluent cells grown in 250 ml (75 cm^2) cell culture flasks were trypsinized with 5 ml of trypsin. Cell suspension was diluted into 1:16 and about 330 µl (1 ml into three wells) of cell suspensions were added to each well.

Cell proliferation assays were carried out in 96 well plates. Confluent cells grown in 250 ml (75 cm^2) cell culture flasks were trypsinized with 5 ml of trypsin. Cell suspension was diluted into 1:66 and 100 µl was added to each well.

Virus Stock

Initial virus stock was obtained by large-scale growth of the HG52 strain of HSV 2. Two sub-confluent 75 cm^2 flasks were infected with HSV 2 (HG52). These infections were carried out in multiplicity of infection (MOI) of 0.001 in infecting inoculums of 7 ml. Incubation for one hour at 37°C was carried out to allow virus adsorption. Following incubation, 25 ml of supplemented media was added. At three days post- infection, the harvested infected cells were transferred to 250 ml Sorvall™ bottles. Virus pellets were obtained by spinning the bottles at 10,000 rpm for 40 minutes at 4°C. The pellet was resuspended in 2 ml of medium. The stock was frozen and thawed once and was vortexed vigorously. The stocks were stored at -70°C in 200 µl aliquots. One aliquot was titrated to determine the virus titer of the stock.

Virus Assay

Viruses were grown in 35 mm dishes in different MOI with different treatment conditions to detect the effect of siRNA on HSV replication (virus burst) in rabbit skin cells or in HeLa cells. These infections were performed on the third day after seeding or 24 hours post-transfection. The dishes were infected with the appropriate multiplicity of infection in infecting inoculums of 500 µl. After the dishes were incubated for 1 hour at 37°C to allow the virus to adsorb, 1.5 ml of supplemented medium were added. The viruses were harvested at the appropriate time periods indicated in the experimental designs.

Titers of virus yields were determined by plaque assay carried in 24 well plates. These plates were seeded with rabbit skin cells as indicated in the cell culture procedure. On the following day, virus infections were carried out in different dilutions. All the titrations were done in triplicates.

Some experiments to detect the plaque forming units were performed in 35 mm dishes. These experiments followed a similar protocol with a few exceptions. Before adding 2 ml of the supplement medium, the infecting inoculums were removed. Three days post-infection the dishes with infected cells were stained using methylene blue and the number of plaques were counted.

Design of siRNA

Gene sequences of HSV 1 and HSV 2 were obtained from NCBI web site with accession NC_001798 for HSV 2 and accession NC_001806 for HSV 1. The Ambion web tool (http://www.ambion.com/techlib/tb/tb_506.html) was used to detect the possible target sites and siRNA sense and antisense sequences. HSV 2 and HSV 1 sequences were

aligned using the NCBI blast "align two sequences (bl2seq)" web tool to identify the homologues gene sequences. The best possible target sites were determined by considering the published criteria.[17,18] Several homologous target sites for siRNA were identified for HSV 1 and HSV 2. Target sites and siRNA sequences for sense and

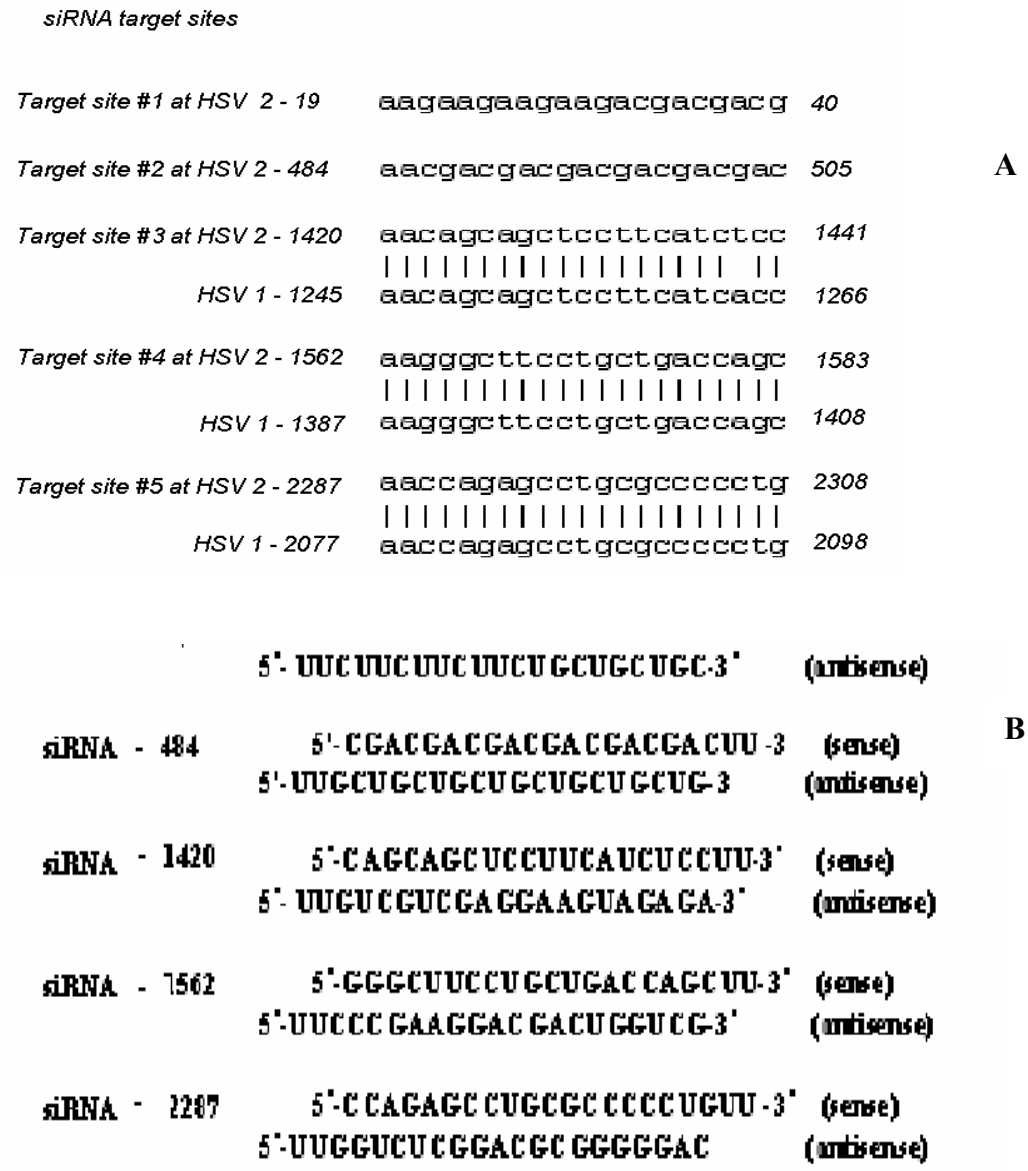


Figure 1. Designing siRNA molecules. A) siRNA target sites. B) Sense and antisense sequences of siRNA molecules.

antisense oligonucleotides are shown in figures 1A and 1B respectively. The siRNA sense and antisense oligonucletides were purchased as 2'-ACE protected RNA from Dharmacon Research (Lafayette, Colorado).

HSV 2 sequence is shown above and HSV 1 sequence is shown below. The number indicates the site of the nucleotide at the ICP4 gene.

Deprotection of siRNA

The tubes containing protected oligonucleotides were centrifuged and 400 μl of 2'-deprotection buffer (100 mM acetic acid) were added. RNA pellets were redissolved by pipetting back and forth. Adequate amounts of RNA oligonucleotides in a deprotection buffer were transferred into a separate tube and the remaining were stored at – 70°C. The samples were vortexed for 10 seconds, spun for 10 seconds, and incubated at 60°C for 30 minutes. Samples were dried using a SpeedVac and resuspended in RNase free water to make 50 μM oligonucleotides samples.

Annealing of siRNA

Equal amounts of sense and antisense RNA were mixed together in 1X siRNA annealing buffer, (6 mM HEPES pH 7.4, 20 mM potassium acetate, 0.4 mM magnesium acetate), and were mixed properly by vortexing. The samples were first denatured by incubating them for 3 minutes at 90°C, and then incubated for 1 hour at 37°C. The annealed samples were stored at -20°C until transfection.

Labeling of siRNA

Cy3 labeled siRNA (Silencer^{TM} siRNA labeling kit- Cy3 from Ambion) was used to detect the optimal condition for the siRNA transfection. 100 μl of reconstitution solution was added to the labeling reagent and mixed well. The labeling reaction was assembled

according to the manufacturer's protocol and incubated for 37°C for 1 hour. Following ethanol precipitation, the RNA pellet was resuspended in nuclease free water.

Transfection of siRNA

Cells were seeded one day before the transfection as indicated in cell culture methods. Fifteen µl of 20 µM siRNA duplex was mixed with 250 µl of Opti-Mem. In a separate tube, 15 µl of Oligofectamine was mixed with 60 µl of Opti-Mem and incubated at room temperature for 10 minutes. The two solutions were combined and incubated for 25 minutes at room temperature. The final volume of 500 µl was obtained by adding 160 µl of fresh Opti-Mem. Finally, without replacing the growth medium, 500 µl of liposome complex was added to each dish and gently mixed by brief rocking.

RNA Extraction

HSV 2 (HG52) were grown in 35 mm dishes as indicated before. The medium was poured off and 1 ml of TriZol reagent was added to each dish. The samples were homogenized by passing the cell lysate several times through the pipette. Following incubation for five minutes at 30°C, 0.2 ml of chloroform was added. Following a vigorous shaking of the tubes by hand, the samples were incubated for 2 to 3 minutes at 15 to 30°C. Samples were centrifuged at 12000 × g for 10 minutes. The aqueous phase was transferred into tubes and RNA precipitation was performed by mixing with 0.5 ml isopropyl alcohol, incubating for 10 minutes at 15 to 30°C, and spinning at 12000 × g for 10 minutes at 2 to 8 °C. The RNA pellet was washed by adding 1 ml of 75% ethanol, vortexing, and spinning the samples at 7500 × g for 5 minutes at 2 to 8°C. The RNA pellets were then air-dried and were redissolved in RNase free water.

DNA was removed from the samples of RNA by DNase treatment (DNA-*free*™ , by Ambion). Briefly, samples were diluted to 100 μg RNA/ml. Then 0.1 volume of 10X DNase I buffer, and 2-3 μl DNase I were added to the samples which were then incubated for 1 hour at 37°C. Five μl of DNase inactivation reagent were added to each of the samples. Following an incubation of 2 minutes at room temperature, the samples were centrifuged at 10000 × g for 1 minute. RNA solutions were then transferred to fresh RNase free tubes.

Reverse transcription was carried out by using the first strand cDNA synthesis kit by Amersham Biosciences. Briefly, RNA samples were heated to 65°C for 10 minutes and then chilled on ice. The uniform suspension of bulk first-strand cDNA reaction mix was added according to the manufacturer's protocol. One μl of DTT solution, and 1 μl of pd $(N)_6$ primer (0.2 μg) were then added to the heat-denatured RNA. Samples were mixed properly by pipetting up and down several times and then incubated for 1 hour at 37°C.

Real time PCR primers and probes were purchased from Applied Biosystems using assay by design. As an endogenous control, eukaryotic 18s rRNA was purchased from Applied Biosystems.

Cell Proliferation Assay

On day one 96 well plates were seeded with rabbit skin cells as indicated in cell culture methods. The serum starvation was performed on day two, after washing the wells with PBS three times. Following another washing with PBS, the treatment medium was added to the wells on day three. Cell proliferation was assayed by using CellTiter 96

Aqueous One Solution cell proliferation reagent (Promega, Madison, WI). Twenty µl were added to each well and the plate was incubated for 1 to 4 hours in 5% CO2 at 37°C.

CHAPTER 4
RESULTS

Assessment of Optimal siRNA Concentration for Transfection

This experiment was designed to determine the optimal siRNA concentration for transfection in HeLa cells. In this study an optimal efficient range for the siRNA transfection was observed to be 100 nM to 200 nM. (Figure 2) Approximately 80% transfections efficiency was observed at the concentrations of 100 nM. . This data was comparable to published data.[3] Further transfections were performed in 100 nM to 150 nM siRNA concentrations.

Evaluation of Effect of Five siRNA on HSV-2 Replication in Rabbit Skin Cells

Five siRNA molecules were designed and purchased from Dharmacon (Lafayette, CO) as indicated in the materials and methods section. The aim of designing five siRNA molecules targeting one gene was to identify the most efficient siRNA and to identify siRNA molecules that have the ability to target both HSV 2 and HSV 1 genes. From here onwards, the siRNA molecules will be termed as siRNA 19, siRNA 484, siRNA 1420, siRNA 1562 and siRNA 2287, where these siRNA molecules target the ICP4 mRNA sequence beginning at nucleotides 19 (siRNA 19), 484 (siRNA 484), 1420 (siRNA 1420), 1562 (siRNA 1562), and 2287 (siRNA 2287) in ICP4 gene of HSV 2. The alignment of ICP4 genes of HSV 2 and HSV 1 revealed that siRNA 1420 and siRNA 1562 and siRNA 2287 should target both HSV 1 and HSV 2 ICP4 genes since the two genes have identical nucleotide sequences at the 22 nucleotides down stream of these positions. The siRNA 1420 has one mismatch with the HSV 1 ICP4 gene. The other two

siRNA molecules, siRNA 19 and siRNA 484 did not share any sequence similarity between HSV 1 ICP4 gene and HSV 2 ICP4 gene.

This experiment was done in quadruplicates. The cells were seeded in 35mm dishes as indicated in materials and methods on day one of the experiment. The following day, the cells were transfected with siRNA at the concentration of 100 nM. Four dishes in one group were treated with Oligofectamine only and were termed the mock group, four dishes in another group treated with Oligofectamine and scramble siRNA, and were termed the scramble group. The remaining four dishes were treated with Oligofectamine and specific siRNA 19, and were termed the siRNA 19 group. Dishes were infected at a low MOI of 0.001 two days after transfection with siRNA, and virus was harvested from the infected cells at 12 hours post-infection. The plaque assay results are shown in Figure 3 and Table1. The difference between virus yields observed in mock transfected group and scramble siRNA transfected group were not statistically significant. However, the virus yields from cells transfected with the five different siRNA molecules revealed a statistically significant reduction in virus replication compared to the scrambled siRNA treated control group. Among all five siRNA treated groups, cells transfected with the siRNA 19 had highest percent reduction (81.7%). Furthermore, the siRNA 484, siRNA 1420, siRNA 1562, and siRNA 2287 showed percent reductions of 45%, 63%, 77.3%, and 68%, respectively, compared to the scramble siRNA control group. These data imply that the five siRNA groups have a specific effect on the reduction of virus replication. Furthermore, it revealed that siRNA 19 was the most effective siRNA. Thus, siRNA 19 was used extensively during further experimental investigations.

Identification of the Best Cell Culture Model to Assess the Effect of siRNA on Reduction of Virus Replication

Our previous experiments showed that siRNA 19 was most effective in reducing production HSV 2 virus in rabbit skin cells. We also wanted to identify the most suitable cell culture model for further experiments. Using the experimental protocol detailed above, a similar experiment was carried out using cultures of HeLa human cervical carcinoma cells to measure the effect of siRNA 19 on HSV 2 replication. As shown in Figure 4, siRNA 19 significantly reduced replication of HSV 2 in HeLa cells by about 72%. This result suggests that the reduction of HSV 2 replication in HeLa cells is almost as effective as previously shown using HSV 2 replication in rabbit skin cells. As rabbit skin cells give a better monolayer for plaque assays, further experiments were carried out in rabbit skin cell cultures.

Effect of siRNA on Plaque Formation

This experiment was carried out to observe the effect of siRNA on the plaque formation of HSV 2 in rabbit skin cells. As shown in Figure 5 and Table 3, a 49% reduction in plaque formation of HSV 2 was exerted by siRNA.

Evaluation of Effect of siRNA 19 on HSV 2 Replication with Different Multiplicities of Infection and Harvested at Different Time Points

Further experiments were carried out by changing the MOI and harvesting virus at different time points. The aim of these experiments was to assess if siRNA 19 was effective in the reduction of virus replication even in higher multiplicity of infections at longer time periods. As shown in Figure 6, it was observed that siRNA 19 was effective in the reduction of HSV 2 replication even in high MOI. These experiments yielded a 78% reduction of virus replication comparing to the mock transfected group and 79% reduction of virus replication compared to the cells treated with scrambled siRNA. These

percent reduction in virus plaque forming units were as high as previously shown values for low MOI. In order to detect the efficiency of siRNA over a longer time period, a similar experiment was carried out with an MOI of 0.01 and virus harvested at 36 hours post-infection. This experiment was expected to show a greater reduction of virus yields with longer harvesting times periods because more cycles of virus replication could occur. As shown in Figure 7 and Table, the number of plaque forming units was reduced about 93% compared with the scramble group. This is the highest reduction in virus yield observed for all the different experimental conditions. This outcome suggests that siRNA effect will last at least for 72 hours post-transfection. Unexpectedly, there was a significantly low yield of virus in the mock transfected group compared to the scramble transfected group. Since the seeding and the infection were all carried out at the same time with the same sample of cell suspensions and virus suspensions, this finding could not be explained by the seeding or infection bias. Considering other possibilities, it was suggested that this effect could be due to poor adsorption of the virus. The stacking of the dishes during incubation might have interfered with even virus adsorption. To prevent this happening in future experiments, the dishes were briefly rocked several times during the 1 hour incubation. This experiment was further extended by harvesting virus at 72 hours (96 post-transfection) and 96 hours (120-hours post-transfection) to observe the lasting effect of siRNA over a longer duration. As shown in Figures 8 and 9, virus yields were reduced at both time points by siRNA 19 compared to the scramble group, but the decreases in virus production were not statistically significant (shown in Tables 5 and 6).

Comparison of Growth of HSV 2 Virus Between Mock Transfected Group, Scramble siRNA Transfected Group and siRNA 19 Transfected Group

The following experiment was carried out with a high MOI of 3, and virus was harvested at 4 hours, 12 hours, 24 hours, and 48 hours. Because of the high MOI, we expected there would be a single burst of virus at the 12 hour post-infection with no additional increase in virus at the subsequent time points (24 and 48 hours). This experiment followed a similar protocol as described earlier. As shown in Figure 11, at 4 hours post-infection, reductions in virus yield were similar for all three treatment groups. This suggests that the different reductions in virus yields in cells treated with Oligofectamine only, scrambled siRNA or siRNA 19 observed in previous experiments were not due to different levels of HSV adsorption. Surprisingly, the virus showed a delayed viral replication rather than giving a single viral burst at 12 hours post-infection. Even more surprising was the fact that the virus yields observed in scramble transfected groups and siRNA 19 transfected groups at 24 hours were lower than the virus yields observed at 12 hours. However, virus replication resumed after 24 hours, producing more virus at 48 hours. Statistical analysis of the data (Table 6) revealed a persistent effect of siRNA on the reduction of HSV 2 replication. However, the reason for the delay in virus replication has yet to be determined.

Cell Proliferation Assay

In this experiment, cell proliferation was measured in cells grown in three different conditions: serum free medium; medium with 5% serum; medium with 5% serum and Oligofectamine; medium with 5% serum, Oligofectamine and scramble siRNA; medium with 5% serum, Oligofectamine and siRNA 19; and medium with 5% serum, Oligofectamine and siRNA 1420. The last four conditions represent the mock transfected,

the scramble transfected, the siRNA #1 transfected groups and the siRNA #3 transfected groups that were discussed in previous experiments. Statistical analysis of the data revealed a significant difference in mock transfected and scramble transfected groups except at the 24 hour time point. Interestingly, at all time points the differences between absorbencies of scramble transfected and siRNAs 19 and 1420 transfected groups were not statistically significant.

A

Oligofectamine +50 nM siRNA-transfection efficiency=49.2%

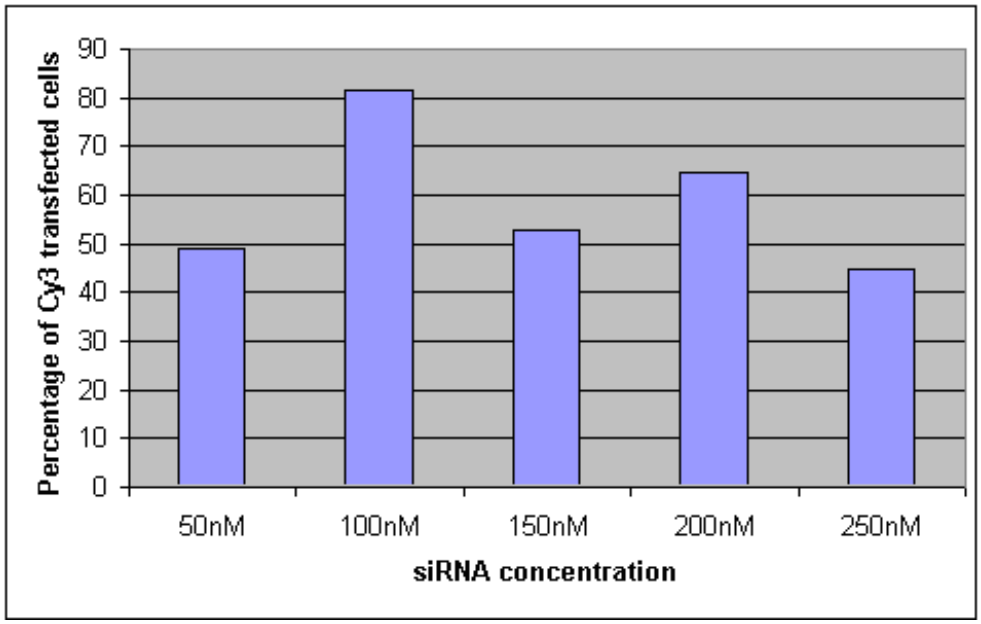
Oligofectamine +100 nM siRNA - transfection efficiency=81.7%

Oligofectamine +150 nM siRNA -transfection efficiency=52.8%

Oligofectamine +200 nM siRNA -transfection efficiency=64.7%

Oligofectamine +250 nM siRNA -transfection efficiency=44.8%

Figure 2. Optimizing transfection conditions. A) Transfection efficiency of siRNA in different siRNA concentration. B) Uptake of liposomal complexes in to the cells in different siRNA concentrations



B

Figure 2. Continued.

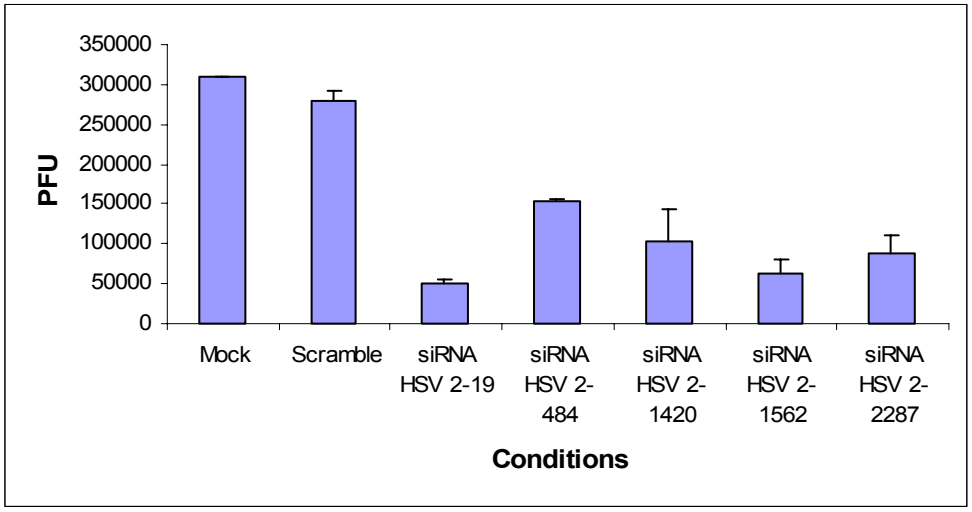


Figure 3. The effect of five siRNA molecules on HSV 2 replication at MOI of 0.001 in rabbit skin cells at 12 hours post-infection.

Table 1. Percent reduction and the P values between siRNAs and scramble

Group	Percent Reduction	P Value siRNA vs. Scramble
siRNA 19	81.7%	0.008
siRNA 484	45%	0.048
siRNA 1420	63%	0.051
siRNA 1562	77.3%	0.004
siRNA 2287	68%	0.009

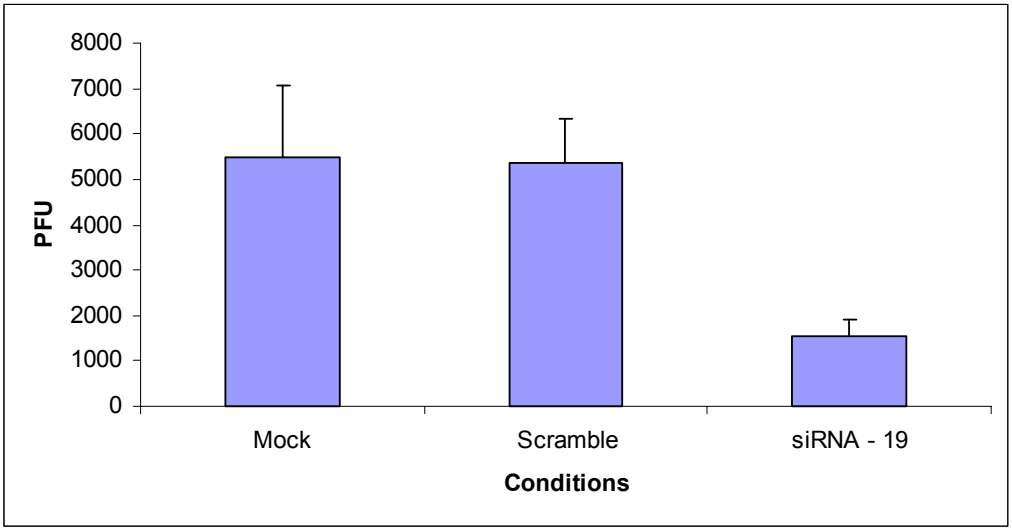


Figure 4. Effect of siRNA #1 on HSV-2 replication grown in HeLa cells

Table 2. Percentage reduction and P values of results shown in Figure 4

Group	**% Reduction** siRNA19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs Scramble	**P Value** Scramble vs SiRNA 19	**P Value** Mock vs SiRNA 19
siRNA 19	71.9%	71.31%	0.009	0.947	0.047

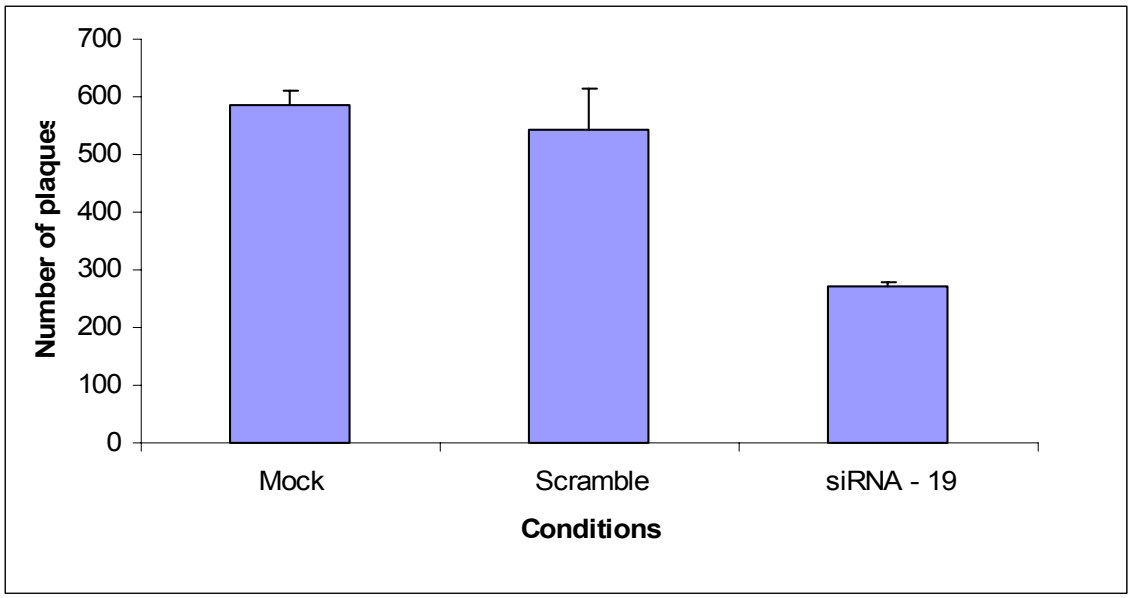


Figure 5. Effect of siRNA on plaque formation of HSV-2

Table 3. Percentage reduction and P values of the results shown in figure 5

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. SiRNA19	**P Value** Mock vs. siRNA 19
siRNA 19	53%	49%	0.606	0.008	0.000035

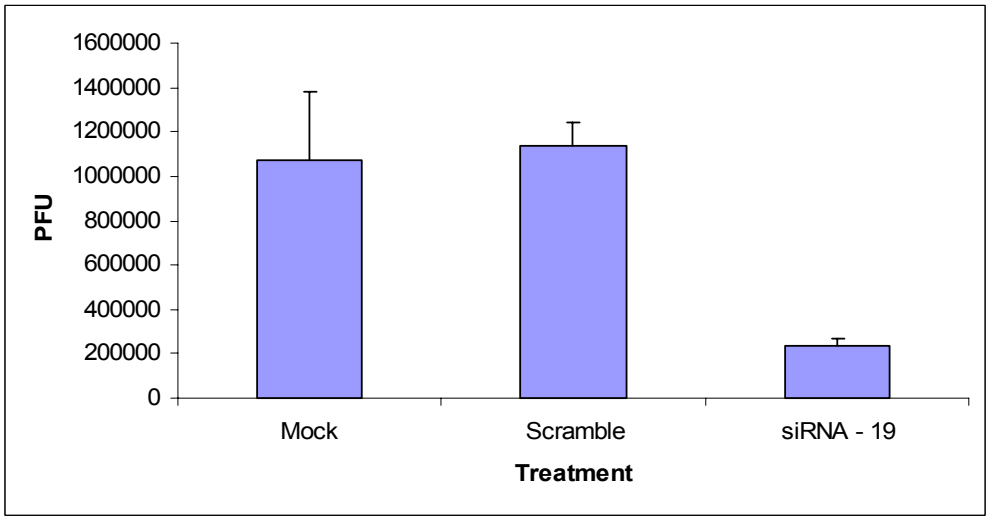


Figure 6. Effect of siRNA on HSV 2 replication at high multiplicity of infection (MOI of 1) infected cells were harvested at 13 hours post-infection.

Table 4. Percentage reduction and P values of the results shown in figure 6

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. SiRNA 19	**P Value** Mock vs. SiRNA 19
siRNA 19	78%	79%	0.86	0.00024	0.035

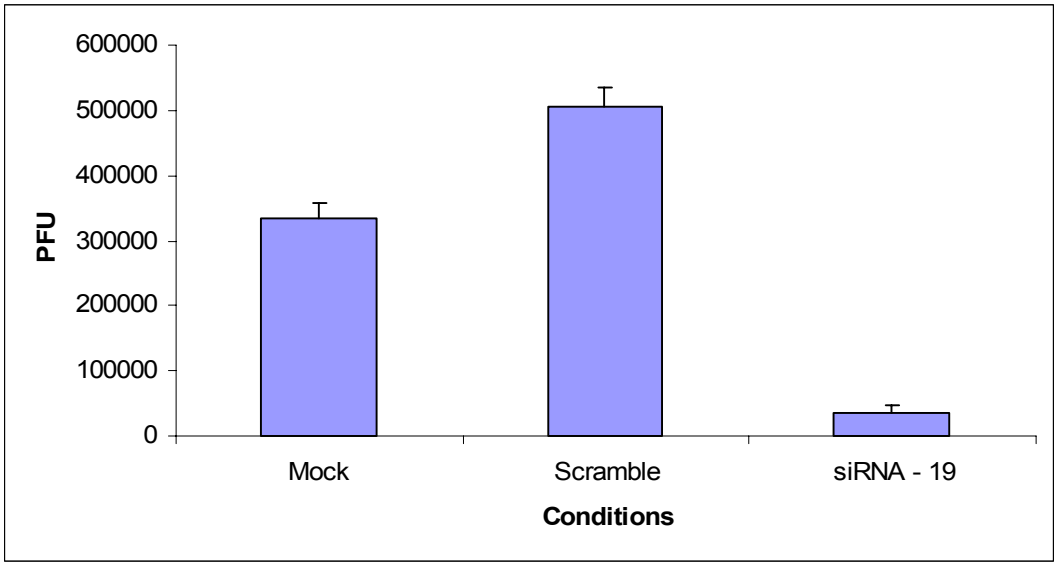


Figure 7. Effect of siRNA 19 on HSV 2 replication with infection of MOI of 0.01 at 36 hours post-infection

Table 5. Percentage reduction and P values of the results shown in figure 7

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. SiRNA 19	**P Value** Mock vs. SiRNA 19
siRNA 19	89%	92.7%	0.04	0.00000701	0.0000174

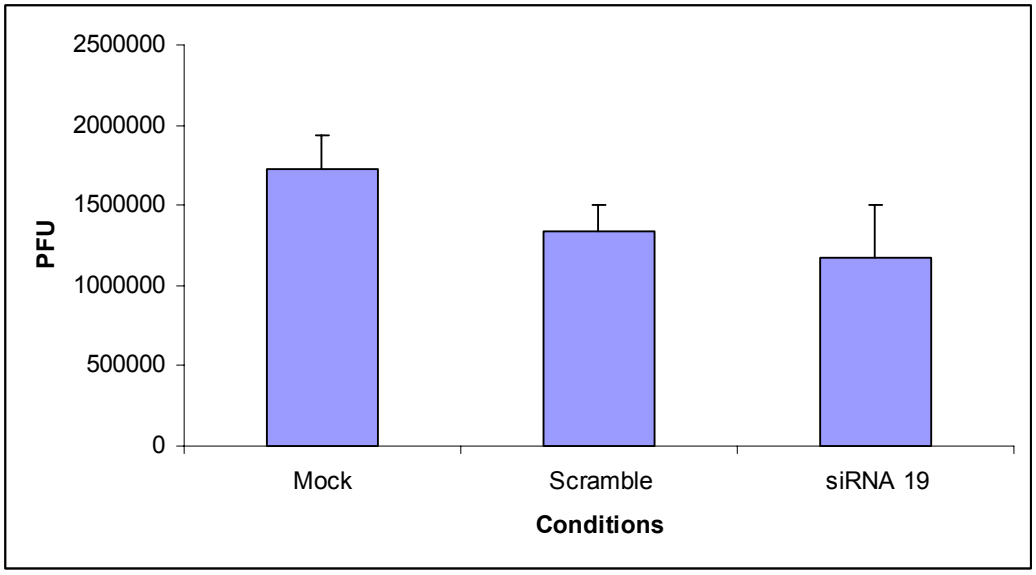


Figure 8. Effect of siRNA on HSV 2 replication with MOI of 0.01 at 72 hours post-infection

Table 6. Percentage reduction and P values of the results shown in figure 8

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. siRNA 19	**P Value** Mock vs. siRNA 19
siRNA 19	32%	12.6%	0.18	0.66	0.21

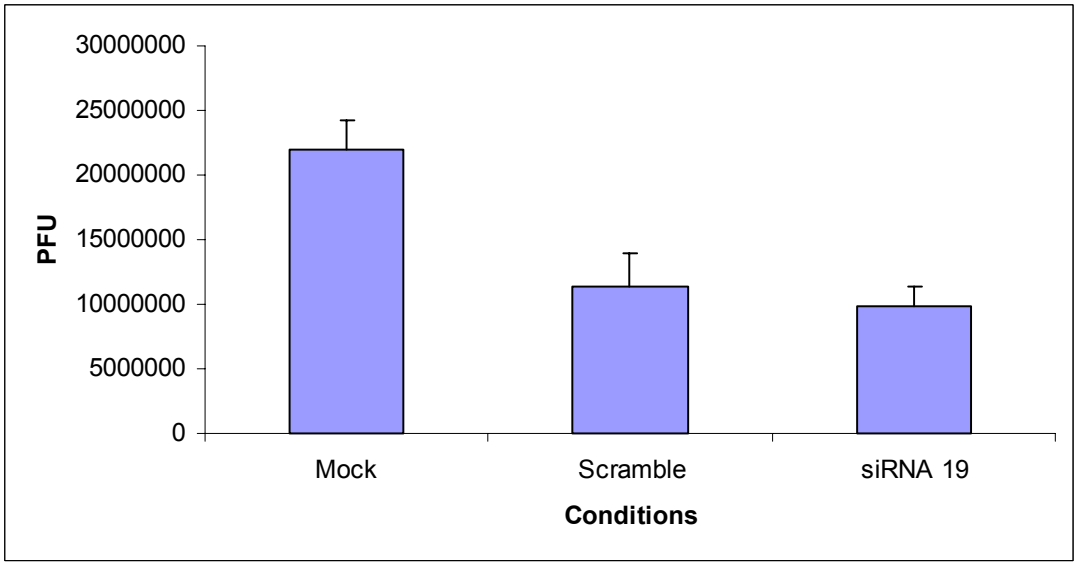


Figure 9. Effect of siRNA on HSV 2 replication with MOI of 0.01 at 96 hours post-infection

Table7. Percentage reduction and P values of the results shown in figure 9

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. siRNA 19	**P Value** Mock vs. siRNA 19
siRNA 19	55%	13%	0.019	0.62	0.003

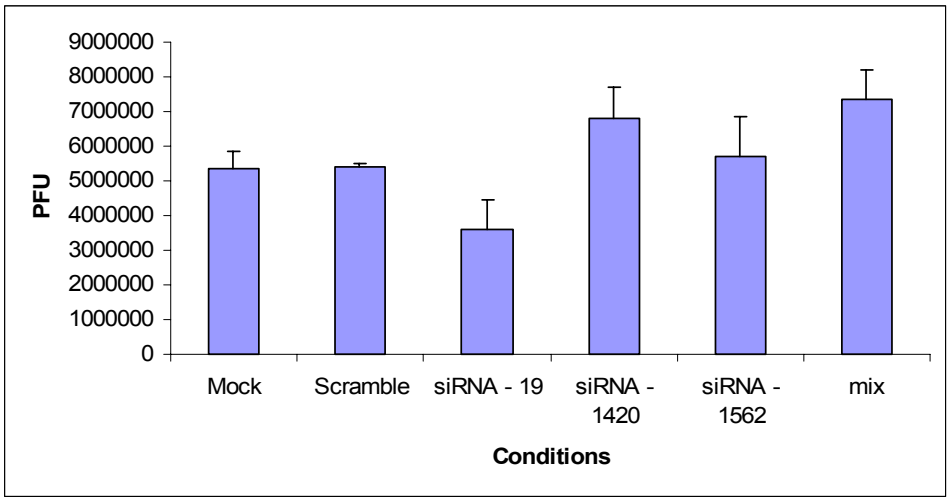


Figure 10. Effect of siRNA on HSV-1 replication in rabbit skin cells.

Table 8. Percentage reduction and P values of the results shown in figure 10

Group	**% Reduction** siRNAs Mock	**% Reduction** siRNA vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. siRNA	**P Value** Mock vs.siRNA
SiRNA 19	32%	32.9%	0.92	0.09	0.14
SiRNA 1420	-	-	0.92	0.206	0.23
SiRNA 1562	-	-	0.92	0.783	0.768
Mix	-	-	0.92	0.087	0.118

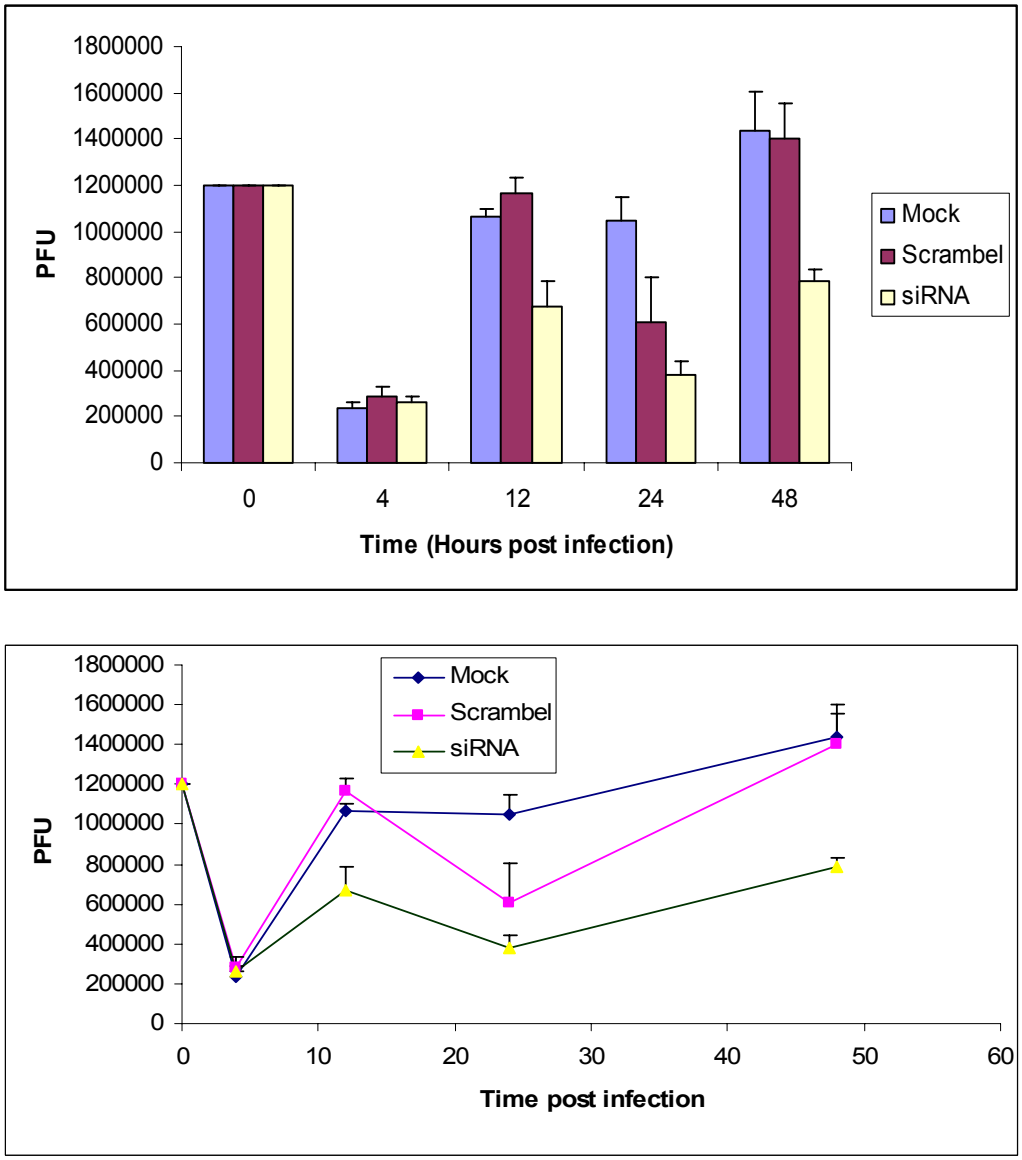
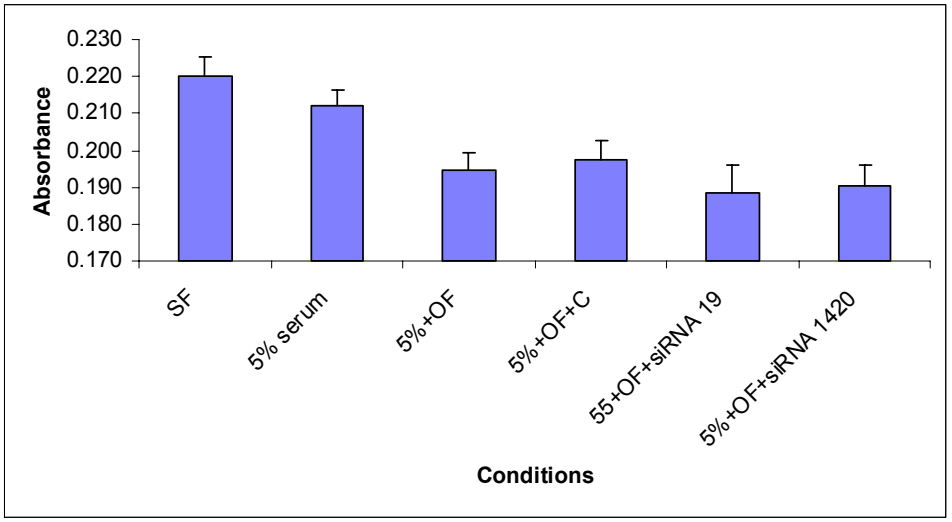


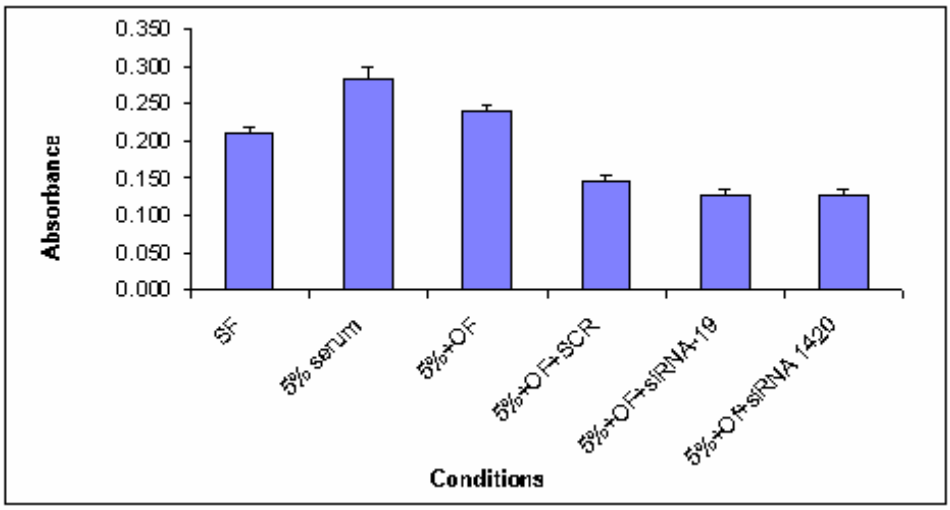
Figure 11. Virus replication assessments. A) Bar graph assessment of virus replication at different time points. B) Line graph assessment of virus replication at different time points

Table 9. Percentage reductions and P values of the results shown in figure 11A

Group	**% Reduction** siRNA 19 vs. Mock	**% Reduction** siRNA 19 vs. Scramble	**P Value** Mock vs. Scramble	**P Value** Scramble vs. SiRNA 19	**P Value** Mock vs. SiRNA 19
4 hours	-	-	0.447	0.689	0.58
12 hours	36.8%	42%	0.28	0.022	0.031
24 hours	64%	37%	0.11	0.33	0.004
48 hours	45%	43%	0.86	0.018	0.018



A



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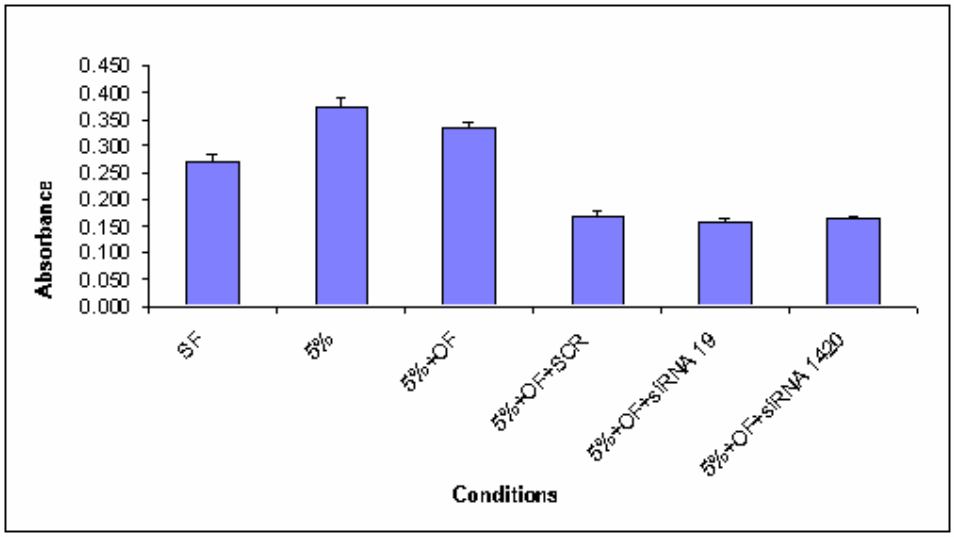

C

Figure 12. Cell proliferation assays at different time points post-transfection. A) 24 hours. B) 48 hours. C) 72 hours.

Figures 12A, 12B and 12C show the cell proliferation assay data. SF indicates that the samples are treated with serum free medium, 5% indicates medium with 5% serum, OF indicates Oligofectamine, C indicates scramble siRNA treatment, 19 indicates siRNA 19 treatment, siRNA 1420 indicates siRNA 1420 treatment.

Table 10. P Values of the data shown in figures 12A, 12B and 12C.

Conditions	**24 hours**	**48 hours**	**72 hours**
Mock vs. Scramble	0.6	0.0000002	0.0000000013
Scramble vs. siRNA 19	0.32	0.18	0.55
Scramble vs. siRNA 1420	0.35	0.03	0.22

CHAPTER 5
DISCUSSION

The Importance of New Therapeutic Measures for HSV

Genital herpes is the most prevalent genital ulcer disease worldwide.[16] Although HSV 2 most commonly causes genital ulcers, the frequency of genital ulcer disease caused by HSV 1 is increasing.[28] Antiviral agents such as nucleoside analogs like Acyclovir and Valacyclovir, guanosine analogs like Penciclovir and Famciclovir, pyrimidine nucleoside analogs like Trifluridine, and adenine analogs like Vidarabine are available as treatments for herpes infections.[9] Along with the oral antiviral agents, topical forms of Penciclovir and Acyclovir are used in patients with recurrent herpes labialis, an ocular formulation of Acyclovir is used for the treatment of ocular herpes, and an Acyclovir ointment is available for use in genital herpes cases. Although these therapies reduce the duration of illness and viral shedding they do not alter the frequency of recurrences. In current practice, the individuals who have frequent recurrences are treated with daily suppressive therapy, but there is no sustained effect after discontinuation of treatment. Clinical studies have revealed an emergence of drug resistance in about 0.3% of immunocompetent hosts and about 4 to 7% in immunocompromised patients with the use of Acyclovir, Penciclovir and their prodrugs over the last two decades.[2] Further, it was observed that the prevalence of Acyclovir resistant HSV is higher in severely immunocompromised patients than in immunocompetent patients.[2,9,43] It was reported that Acyclovir resistance is mainly due to thymidine kinase mutations, and less frequently, due to mutations in viral DNA polymerase.[9,26,25,34] These mutations were

identified to result in decreased or absent HSV thymidine kinase production, altered affinity of the thymidine kinase for Acyclovir-triphosphate, or altered affinity of the HSV DNA polymerase for acyclovir-triphosphate.[9,37] Although the reactivation of Acyclovir-resistant HSV may still be rare and unrecognized in immunocompetent people, new therapeutic and preventive strategies for HSV disease are needed to prevent the rise of drug resistant HSV. Although it has been demonstrated that the utilization of higher doses of Acyclovir for longer periods of time has improved the outcome of neonatal HSV disease, morbidity and mortality from neonatal HSV infections remain unacceptably high.[25] As there is an increasing prevalence in immunocompromised population due to the improvements in life expectancy for the immunocompromised, for example, people in immunosuppressive therapy with organ transplants and patients with AIDS, the maintenance of life-long prophylactic treatment to prevent HSV recurrences could significantly impact the cost of patient care. Secondly, there is a need for a new treatment strategy to overcome the emergence of drug resistant HSV infection. Since neither of the available antiviral therapies cures the latent herpes infection there is a significant need for a new treatment strategy that targets the replication mechanism of the virus at the transcriptional or post- transcriptional level.

Feasibility of RNA Interference as a Therapeutic Measure in HSV Infection.

siRNAs are typically 21nt double-stranded molecules that specifically cleave mRNA leading post-transcriptional regulation.[17] Although siRNAs are known to be highly specific it has recently been shown that siRNAs are effective in single mutations located in the center of molecules, and up to four mutations are required for complete inactivation.[17,10] Spontaneous or drug induced mutations are known to be the major cause of drug resistance to many viruses. Hence the ability to tolerate one or few mismatches

makes siRNA an appealing strategy for HSV treatment. siRNA molecules could be delivered either exogenously or endogenously. Exogenous delivery offers the possibility of designing therapeutic siRNAs for acute HSV infections only when the siRNAs need to be delivered immediately and should be active only for a short duration. Recent studies have shown that short hairpin siRNAs can be expressed in plasmid or viral vectors such as adenoviral and lentiviral. These short hairpin siRNAs were shown to express for longer durations. This quality of siRNA offers the possibility to chronically express the therapeutic siRNA in host, which will inhibit the recurrence of herpes infections. siRNAs have been shown to be effective in decreasing virus production in HIV-1[11,22,30,33,42], Hepatitis C[24], Human papilloma virus[31] and Hepatitis B virus.[23] Furthermore, it was shown that short hairpin siRNA expressed in plasmid or virus vectors can exert a sustained inhibition of viral replication.

How is This Study Designed to Overcome the Prevailing Drawbacks in Current Treatment Methods?

In order to overcome the antiviral resistance to thymidine kinase and viral polymerase, we designed and tested siRNA molecules that target an essential immediate early gene, ICP4. In order to address the possible failure in siRNA treatment due to virus mutations that could occur in the ICP4 gene, five siRNA molecules were designed and shown to be effective in the reduction of HSV 2 replication. Statistical analysis of this data revealed a significant reduction in virus yields in the samples treated with siRNA molecules. Three siRNA molecules, siRNA 1420, siRNA 1562,and siRNA 2287, were designed to target the identical gene sequence region observed between HSV 1 and HSV 2. Here we suggest that these areas of the ICP4 gene carry less of a chance for mutation. Further, this approach would enable the design of a siRNA that could target

both HSV 1 and HSV 2 viruses. Surprisingly, the results observed in the experiment on HSV 1 using siRNA 1420, siRNA 1562, and siRNA 2287 did not reveal any effectiveness on the reduction of HSV 1 replication. However, the same siRNAs had a promising effect on the reduction of HSV 2 replication. This finding suggests that the siRNA function might be affected by the secondary structure of target mRNA. This suggestion contradicts the previous published data that states that siRNA function is not affected by the secondary structure of mRNA.[18] It is also possible that the high MOI used with the HSV 1 experiment over whelmed the effect of the siRNA. This concept could be tested by performing the experiment with a low MOI of HSV 1 of 0.001.

Effects shown in HeLa cells of virus replication show a significant reduction on HSV 2 replication by siRNA 19. This suggests the ability of siRNA 19 to reduce HSV 2 replication in human cells.

In this study we preformed an experiment to test the effect of siRNA on plaque formation of HSV 2. Usually relatively healthy cells are needed to obtain a monolayer, in which the plaques can be preformed. Although siRNAs have been reported to have minor toxicity on the cells (off target effects), our results (Figure 5 and Table 3) suggest that any nonspecific siRNA effects did not influence the formation of a monolayer. Further studies shown in Figures 6, 7, 8, 9, and 11 were carried out to detect the siRNA effect on different MOI harvesting at longer time points (24, 36, 48, and 72, and 96 hours). These studies revealed a sustained successful effect of siRNA over reduction in virus replication over at least 72 hours post-transfection. A trend of increased reduction of virus replication with time was observed until the infection reached 72 hours post- infection or more. This trend was expected, as HSV 2 is usually known to have continuously rising

virus titers. The reduction in virus yield at the first burst should give rise to an even more reduced virus yields at late virus bursts. However, there was no effect of siRNA shown after 72 hours post-infection. Further, it was shown that the difference between mock transfected, scramble transfected, and siRNA 19 transfected groups observed in 76 hours post-transfection experiment are not statistically significant. The inability of siRNA to reduce the virus yield at 72 hours post-infection (i.e., at 96 hours post-transfection) could be explained by the waning effect of siRNAs at longer durations. The results suggests that the reducing effect of siRNA in survived cells in the siRNA 19 transfected group allow a reinfection leading to unopposed virus replication. Since previous literature has shown a sustained effect in short hair pin siRNA[8], in this study we would suggest that these siRNA should be expressed as short hair pin RNA in a plasmid or in a virus vectors in which these siRNAs can be expressed longer and have a better effect on the inhibition of virus replication.

Although the results discussed above support the conclusion that designed siRNA molecules significantly reduce HSV 2 replication, further experiments were carried out to exclude other possibilities that could also give rise to low virus yields. It was speculated that these low yields might be due either to low virus uptake, or due to nonspecific effects of siRNA. Here we have addressed the low virus adsorption issue by showing an ecliptic effect of HSV in all three conditions in experiments shown by virus growth curve. We expected to have a single large virus burst around 12-18 hours post-infection followed by a plateau state in the growth curve detection experiment carried out with a high (MOI 3) multiplicity of infection. Surprisingly, it was observed that a delay in virus production in all three groups and virus titers varied substantially by dipping at 24 hour post-infection

in Scramble transfected and siRNA 19 transfected groups. Previously, it was shown by S J Su et al that HSV 1 exhibits a substantial variation in virus yield at different time points. {2688} But they have also shown a continuous rise in HSV 2 infected cells. These findings were in contrast with the results of Chen j et al, who observed a continuous rise in virus titer in their growth curve experiments.[16] Further, S J Su et al. found that the cytopathic effect of both HSV 1 and HSV 2 were almost the same in the last stages. But this study showed a delay in virus replication. This could be due to some effect exerted by Oligofectamine. It was interesting to observe a similar pattern in both scramble treated and siRNA 19 treated groups, which showed a fluctuation in virus growth. Since both siRNA groups showed a similar pattern it was suggested that these results could be due to a nonspecific effect of siRNA. This finding is in accordance with published data that show the off-target effect of siRNA.[40,41] These nonspecific effects could be affecting virus growth itself or could be due to a change in the environment of virus growth such as cell death which causes less viable cells that, in turn, give rise to reduced virus replication. If the nonspecific effects of scramble treated and specific siRNA treatment cells are shown to be equal, the scramble group would still be a good control for analyzing data.

The CellTiter 96 Aqueous one solution cell proliferation assay usually detects metabolically active cells. In this study we have assessed the metabolically active cells grown in different treatment groups. It was found that there was a reduced number of viable cells in all the groups that were treated with Oligofectamine. This finding suggests that Oligofectamine affects cell growth. Since all the virus yield were compared with controls containing Oligofectamine, these results would not affect the previous

conclusions of this study. Although the absorbance of siRNA treated groups seems to be lower compared to scramble treated groups, statistical analysis revealed a non significance in such a finding. Hence, it can be concluded that the difference in the number of viable cells in different treatment groups does not affect the virus yields observed in previous experiments. These findings agree with previous published data showing that siRNA do not activate the IFN response.[12]

We began this study with the hypothesis that siRNA molecules can be used to reduce virus growth by specifically targeting the ICP4 gene. Analysis of data showed that all five siRNA molecules could be used to reduce the virus growth of HSV 2. However, analysis also showed that siRNA 1420, siRNA 1562, and siRNA 2287 do not effectively inhibit the HSV 1 virus replication.

Here we suggest performing a real time PCR, which would asses the quantitative reduction of the ICP4 mRNA. This study promises the possibility developing siRNA as a therapeutic measure. Further, we would suggest designing new siRNA molecules using the latest published criteria that would target ICP4 of both HSV 1 and HSV 2.

CHAPTER 6
CONCLUSION

This study shows a specific reduction of virus replication using siRNA molecules that target ICP4, an essential immediate early gene of herpes simplex virus type 2. It was predicted that the siRNA molecule could reduce the virus replication in cell culture models. All the data shown in this study exhibited a statistically significant reduction in virus growth. The reduction of virus yields observed in this study shows a maximum possible reduction of siRNA considering the transfection efficiency and lifetime of siRNA molecules. The ability to reduce virus growth in higher multiplicity of infections suggests that the siRNA concentration used in this study would be sufficient to counteract such a high dose of infection. The inefficiency of siRNA molecules in latter time points of post-transfection indicates the importance of endogenous expression of siRNA or the importance of multiple doses of siRNA in therapeutic use. The ability of siRNA molecules to function well in rabbit skin cells predicts a success in testing these siRNA in rabbit skin models. Further, the efficiency shown in HeLa cell suggests the feasibility of using these siRNAs in human culture cell model and also developing this as a therapeutic strategy to treat herpes infections in humans.

In addition, this study addresses the nonspecific effects of siRNA molecules in the cell proliferation assay. This data suggests that Oligofectamine and siRNA molecules exert a nonspecific toxic effect. However, this does not affect the conclusions made before, because these conclusions were made comparing the virus yields of scramble group and siRNA group. All these findings strongly suggest that siRNA could be used as

a promising therapy in the cure of the latency of herpes infection and also could be used as a valuable strategy for the treatment of resistant herpes simplex viruses.

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BIOGRAPHICAL SKETCH

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