Antiviral activity of enzymatically synthesized siRNA swarms against herpes simplex virus type 2 clinical strains

Pro gradu-thesis
University of Turku
Department of Biochemistry
Digital Health and Life Sciences,
Molecular Systems Biology
06/2021

Huda Habib
Supervisors: Veijo Hukkanen, Marie Nyman

Abstract

HSV-1 and HSV-2 are closely related human pathogens that share approximately 50% overall sequence homology and cause latent infections recurring as orofacial and genital herpes. Recently a novel treatment modality was introduced against HSV-1 utilizing enzymatically synthesized pools or swarms of siRNAs. These siRNA swarms trigger the RNAi pathway and inhibit viral replication by silencing essential viral genes. Viruses are ideal targets for RNAi therapies. For example, siRNA drugs can be developed more rapidly than small inhibitory drugs to combat newly emerged viral strains or escape mutants. The antiviral siRNA swarms used in this study target the essential HSV-1 genes *UL27*, *UL29* and *UL54*. My aim was to determine whether these same swarms could silence the corresponding HSV-2 genes, inhibiting viral replication. The effects of swarms were studied on several HSV-2 clinical isolates in mink lung cells (MLCs) and human keratinocytes (HaCaT). Plaque titration was used to establish the viral titer in plaque forming units and qPCR was performed for the HSV-2 *gD* gene to determine the genome copy number. All anti-HSV swarms reached significant inhibition levels in MLC, the UL29 swarm being the most effective. Inhibitory effects were present but less pronounced in HaCaT cells.

Table of Contents

1 Review of literature

1.1 Herpes simplex viruses

Herpes simplex viruses (HSVs) are human pathogens that belong to the Herpesviridae family. There are two species of these viruses, herpes simplex virus 1 and 2 (HSV-1 and HSV-2), which mainly cause oral and genital herpes respectively. (Whitley and Roizman 2001; Hukkanen and Seppänen 2020.) These viruses are closely related and share 46% overall sequence homology with 85% identical nucleotides (Kieff et al. 1972). It is believed that HSV-2 evolved from a chimpanzee herpes virus whose predecessor diverged from HSV-1 six million years ago. The chimpanzee herpes virus then returned to the human lineage two million years ago and became what is known today as HSV-2. Because HSVs are old, they have evolved closely together with their human hosts and are extremely well adapted to them. (Hukkanen 2020.)

HSV-1 and HSV-2 consist of a large double stranded DNA genome surrounded by an icosapentahedral capsid, an amorphous protein layer called a tegument and an outermost envelope (Fig. 1) (Grünewald et al. 2003). Their genome is approximately 152 000–155 000 base pairs long and consists of two covalently linked DNA segments with unique sequences designated as U_L (unique long) and U_S (unique short). These segments are flanked by inverted repeats. (Hayward et al. 1975; Widener and Whitley 2014.)The genome codes for at least 84 proteins which can have several functions (Ward and Roizman 1994). HSV needs all of its genes when replicating in different organs and establishing a recurrent infection (Hukkanen and Seppänen 2020).

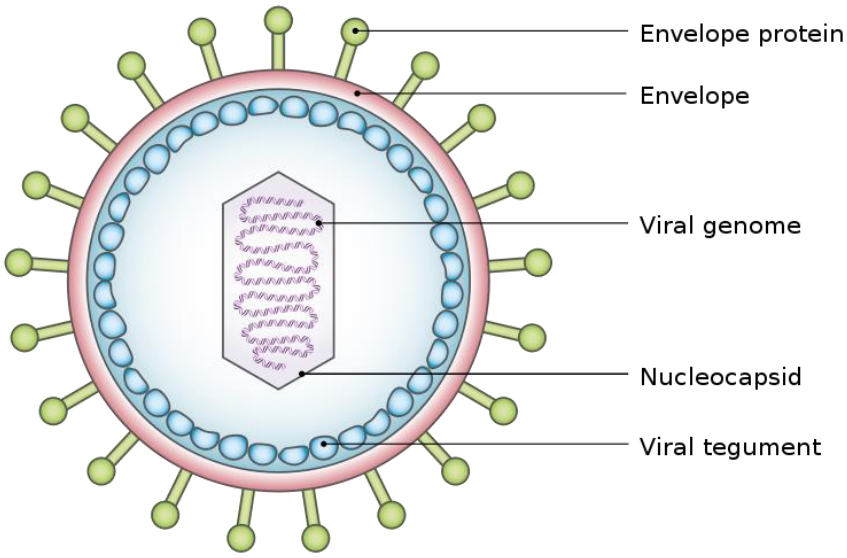

Figure 1. The structure of HSV. *The viral genome consists of double stranded DNA contained in an icosapentahedral capsid. The capsid is surrounded by amorphous proteins which make up the tegument. The tegument is enclosed by an envelope which contains several envelope proteins. (Taylor 2010)*

HSV-1 and HSV-2 cause latent infections during which they stay dormant in their host for up to decades before they reactivate and cause a new infection (Hukkanen 2020). HSV-1 stays latent in the trigeminal ganglia while HSV-2 establishes latency in the sacral ganglia (Widener and Whitley 2014). Latency not only increases the pathogenicity of the virus (Whitley and Roizman 2001), but it also allows the virus to remain in the human population by giving rise to predominantly mild, non-lethal infections (Hukkanen 2020). This in turn has made viral transmission from generation to generation effortless (Hukkanen 2020).

Another distinguishing factor of HSV is its neurovirulence, the ability to infect and replicate in neural tissue. Neurovirulence is mediated by the $\gamma_1$34.5 protein whose function affects Eukaryotic translation initiation factor 2A (eIF-2A) despite the presence of active Protein kinase R (PKR). As a result, even though active PKR may phosphorylate eIF-2A to stop protein synthesis, eIF-2A remains dephosphorylated due to the function of $\gamma_1$34.5. This allows protein synthesis to continue in neuronal cells, which gives the virus enough time to replicate in the cell before it is eventually destroyed. (He et al. 1997; Hukkanen and Seppänen 2020.) In addition to neurovirulence, the $\gamma_1$34.5 protein plays a role in preventing cellular defences such as interferon (IFN) induction, autophagy and xenophagy (Hukkanen and Seppänen 2020).

HSV infections are commonly treated with acyclovir, a synthetic acyclic purine-nucleoside analogue, either topically, orally or intravenously (Whitley and Roizman 2001). HSV can also be treated with precursor drugs valaciclovir and famciclovir that have better oral bioavailability (Balfour 1999). While acyclovir and its precursor drugs can ease disease symptoms and lower recurrences of genital herpes when taken orally, these drugs cannot fully prevent viral reactivation (Whitley and Roizman 2001). Additionally, there are currently no vaccines available for either virus, though several potential HSV-2 vaccines are undergoing development (Johnston et al. 2016).

1.1.1 Herpes simplex virus type 1

HSV-1 mainly causes orofacial herpes with an incubation period of 2 – 12 days (Whitley and Roizman 2001). The virus is transmitted through close contact of mucosal surfaces. A primary infection occurs when a person seronegative to either HSV-1 or HSV-2 develops an HSV infection. A recurrent infection on the other hand describes an infection that is periodically recurring in an HSV seropositive individual. (Whitley et al. 1998.) While a primary HSV-1 infection may be asymptomatic, some common symptoms of a primary infection include a lesion around the mouth area (Fig. 2), swelling of surrounding lymph nodes and fever. The disease usually heals in roughly a week and reoccurs at varying frequencies depending on the individual. Lack of symptoms during a primary HSV infection does not preclude latency. (Hukkanen and Seppänen 2020.)

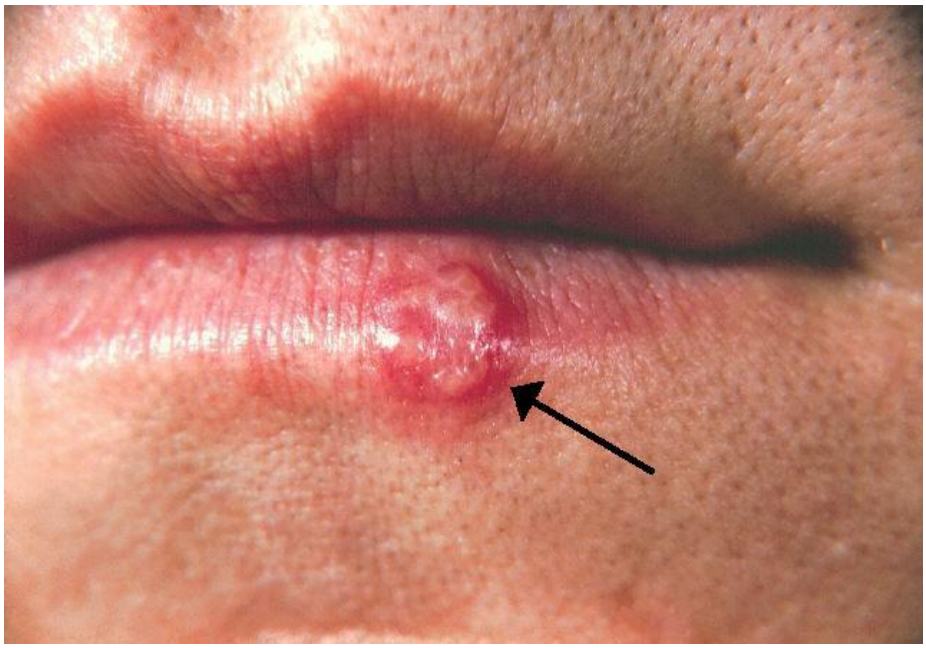

Figure 2. A herpes lesion on the lip caused by HSV-1. *Infections by HSV-1 may be asymptomatic but common symptoms include lesions around the mouth area which typically heal within a week. The disease reoccurs at varying frequencies depending on the individual. (Centers for Disease Control and Prevention 2010)*

HSV-1 infections occur in both developed and developing countries. The prevalence of the virus is affected by several factors such as geographic region, age and sex. In 2016, prevalence was the highest in the South-East Asia region for oral HSV-1 infections and the Americas followed by Europe for genital HSV-1 infections. During the same year, in the global population aged 0 – 49, 3,6 billion people, or an equivalent of 63,6% of the global population, harboured an oral HSV-1 infection while 192 million people aged 15 – 49 or an equivalent of 5,2% of the entire population had a genital HSV-1 infection. The number of people infected with both oral and genital HSV-1 increased with age. (James et al. 2020.)

Aside from orofacial herpes, an increasing number of genital herpes infections are also caused by HSV-1 (Tuokko et al. 2014; Looker et al. 2015). This increase is hypothesized to be caused by changes in sexual behaviour. Oral sex has likely increased in frequency and this, coupled with the overall decrease in HSV-2 genital herpes cases offers one explanation for the increase in the number of HSV-1 genital herpes cases. (Hukkanen and Seppänen 2020.)

Oral and genital herpes are the most common diseases caused by HSV-1 but transmission of the virus can also lead to infections in ocular tissue (Rowe et al. 2013) and the central nervous system (CNS) or to generalized infection in neonates or immunocompromised individuals (Hukkanen and Seppänen 2020). Ocular tissue infection can occur in the lids, cornea, retina, uveal tract and conjunctiva (Rowe et al. 2013). Conjunctivitis with possible inflammation of the eyelids and corneal epithelial dendritic lesions are typical manifestations of primary ocular infections which occur rarely and mostly in the younger population (Darougar et al. 1985). More commonly ocular infections result from viral reactivation of latent infection in the trigeminal ganglia. When this reactivation occurs in the ophthalmic branch of the trigeminal ganglion viral shedding can occur in the corneal surface resulting in infectious epithelial keratitis (IEK) or herpes stromal keratitis (HSK). (Shimeld et al. 1990; Rowe et al. 2013.) The difference between the two, is that lesions are restricted to the corneal epithelium in IEK, while in HSK lesions can appear in the stroma or the overlying epithelium (Darougar et al. 1985). Viral replication in the epithelium results in epithelial cell destruction. Overall, HSV-1 caused recurrent keratitis can manifest as HSK, epithelial dendritic

lesions and geographic epithelial lesions (Labetoulle et al. 2005). HSK is especially problematic because the risk of disease recurrence is significantly increased by previous instances of infection. Additionally, HSK recurrence may result in irreversible corneal damage and eventual blindness. (Wilhelmus et al. 1998.)

HSV infections in the CNS include herpes simplex encephalitis (HSE) and neonatal herpes (discussed more in the chapter Herpes simplex virus type 2) which generally occur at the age of over 3 months or under 1 month respectively. Both infections cause severe neurologic damage and can lead to death. (Widener and Whitley 2014.) HSE is most commonly caused by HSV-1 (Hukkanen and Seppänen 2020) and leads to brain tissue necrosis by causing inflammation, congestion and haemorrhages (Widener and Whitley 2014). Patients may experience disorientation, changes in behaviour and seizures. Approximately one third of HSE cases are caused by primary infection while the rest are attributed to recurrent infection. The resulting infection can travel to the brain through the trigeminal or olfactory nerve pathways. Even when the infection is cleared patients may be left with lasting sequelae such as dementia, learning and memory difficulties. (Hukkanen and Seppänen 2020.) Some less dangerous manifestations of HSV caused CNS infections include HSV aseptic meningitis and radiculopathies (Widener and Whitley 2014).

1.1.2 Herpes simplex virus type 2

HSV-2 infections most commonly lead to genital herpes, a disease transmitted through sexual contact. The incubation period of HSV-2 is the same as for HSV-1, ranging from 2 – 12 days. Symptoms for primary genital herpes include macules and papules as well as vesicles, pustules and ulcers. Recurrent infection in males causes 3 – 5 vesicles in the penis shaft while in females symptoms appear as ulcerating-vesicle lesions in the genital area or as vulvar irritation. Absence of symptoms does not prevent the virus from being transmitted from one person to another as virus shedding occurs even in asymptomatic infection. (Whitley and Roizman 2001.) Typically, recurrent infection causes milder symptoms that can last 1 – 2 weeks. Additionally, recurrence of genital herpes depends on the virus causing it, as genital herpes caused by HSV-2 reoccurs at higher frequencies than genital herpes caused by HSV-1. (Hukkanen and Seppänen 2020.)

HSV-2, like HSV-1, occurs worldwide in both developed and developing countries (James et al. 2020). Because HSV-2 transmits sexually, seroconversion usually only occurs once a person has

become sexually active (Whitley and Roizman 2016). Geographical region, sex and the number of sexual partners all play a role in HSV-2 acquisition. Females are more likely to contract the virus than males, presumably because they have more mucosal surface. (Widener and Whitley 2014.) In 2016 an estimated 491,5 million people worldwide aged 15 – 49 were infected with HSV-2, which equates to 13,2% of the global population. Prevalence was the highest in the African region and the number of infected individuals increased with age though this number was also affected by differences in population sizes. Additionally, the highest prevalence across all regions was observed in women. (James et al. 2020.)

HSV-2 significantly increases human immunodeficiency virus (HIV) transmission as the risk to acquire HIV is up to threefold higher when a person is infected with HSV-2 (Looker et al. 2017). This is because ulcers, even microscopic ones, caused by genital herpes allow HIV to enter the body and cause infection. Ulcers caused by herpes also lead to increased levels of CD4 (cluster of differentiation 4) lymphocytes that HIV can then target and destroy. (Wald and Link 2002.) Additionally, HSV-2 is able to upregulate HIV replication which in turn increases HSV-2 recurrence, creating a vicious cycle for infection (Margolis et al. 1992; Widener and Whitley 2014).

Much like HSV-1, infections by HSV-2 can lead to infections in the CNS, especially in neonates and in immunocompromised individuals. Neonatal herpes is predominantly caused by primary HSV-2 infections and typically transmission occurs through the birth canal from either symptomatic or asymptomatic genital herpes. When the causal agent of infection is HSV-1, neonatal herpes only occurs during primary infection. Rarely is neonatal herpes caused by an intrauterine HSV infection. (Hukkanen and Seppänen 2020.) The development of neonatal herpes is dependent on the timing of the mother's HSV acquisition. Thus, a newborn is least likely to contract HSV when the mother acquires an HSV infection prior to labour and the chance increases when the mother's acquisition of infection occurs near labour. (Brown et al. 1997.) Overall, the majority of neonatal herpes infections occur during the peripartum period, followed by the postpartum period (Widener and Whitley 2014).

Neonatal herpes can also be classified based on symptoms. The SEM form of disease is characterised by lesions on the skin, eyes or mouth. Disseminated disease involves multiorgan infection that causes newborns to experience lethargy, irritability, seizures and respiratory distress among other symptoms. The organs involved include the skin, mouth, eyes, lungs, liver, adrenal gland as well as the CNS. Disseminated disease can be accompanied by encephalitis or manifest on its own as neonatal herpes. Encephalitis shares many of the same symptoms as disseminated

disease along with temperature instability, poor feeding and bulging fontanel and pyramidal tract signs. (Widener and Whitley 2014.) Neonatal herpes can be prevented by a caesarean section but more commonly transmission is prevented by giving the mother an antiviral prophylactic before labour. Additionally, antibodies gained from the mother help relieve the symptoms of the newborn. (Hukkanen and Seppänen 2020.)

1.1.3 Herpes simplex virus infection biology

HSV infects and attaches onto cells through glycoproteins B, C and D (gB, gC and gD). Glycoproteins B and C initiate the attachment by interacting with cell surface heparan sulfate while gD binds to the HSV entry receptor. Out of all HSV receptor molecules HVEM (herpesvirus entry mediator) and HveC (herpesvirus entry mediator C) act as receptors for both HSV-1 and HSV-2 whereas HveB (herpesvirus entry mediator B) is mainly an HSV-2 receptor. (Hukkanen and Seppänen 2020.) HveC and HveB which are also known as Nectin-1 and Nectin -2 respectively belong to the immunoglobulin G superfamily and both act as intracellular adhesion molecules (Campadelli-Fiume et al. 2000). After binding to an HSV receptor, the envelope of the virus fuses with the cellular plasma membrane and the capsid is transported along microtubules to the nucleus (Fig. 3). The viral DNA is released into the nucleus through the nuclear pore. Gene expression takes place in three phases and is initiated by VP16 (αTIF), an HSV transactivator protein, which enters the nucleus and initiates the transcription of alpha (immediate early) genes. The cellular transcription factors and other proteins are also involved in transcription initiation. (Roizman and Whitley 2013; Hukkanen and Seppänen 2020.) Expression of alpha genes yields alpha proteins that initiate the transcription of beta (early) genes. Beta proteins then initiate the viral DNA replication, which enables the expression of the final set of gamma or late genes. (Hukkanen and Seppänen 2020.)

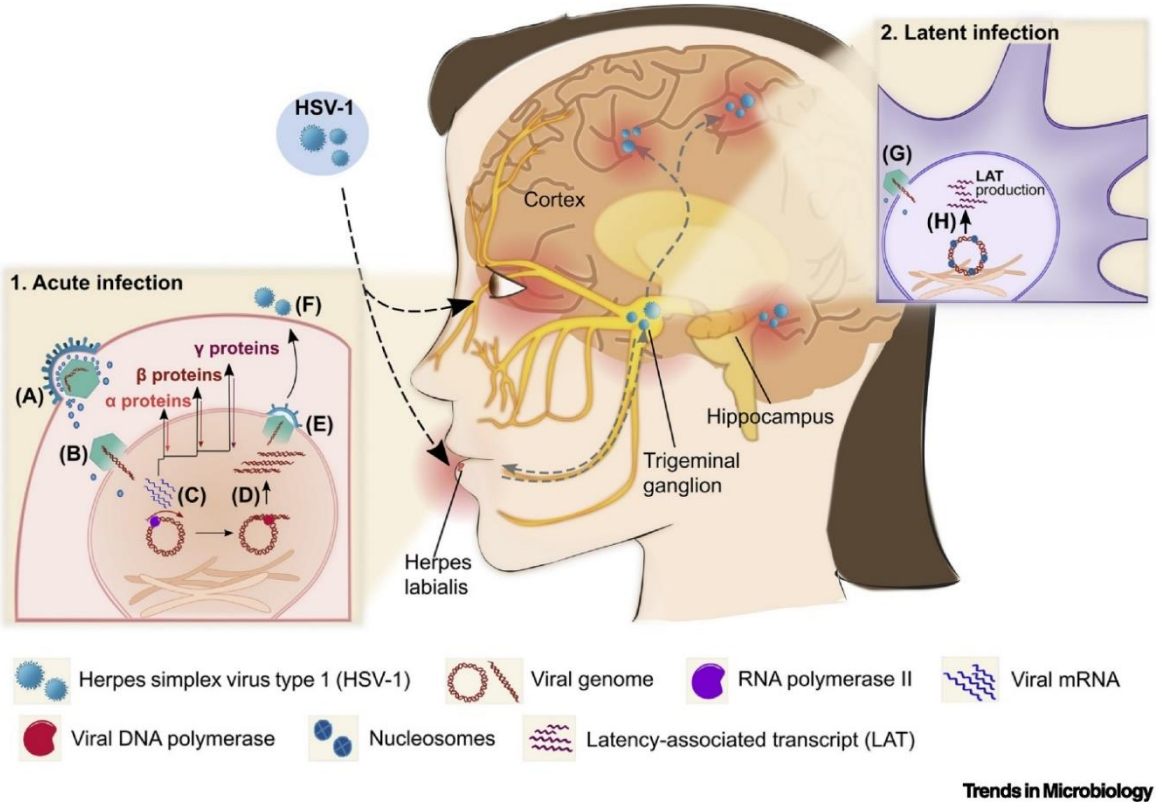

Figure 3. The different stages of acute and latent infection by HSV-1. *In acute infection (1.) the virus binds to an HSV receptor after which the viral envelope fuses with the cellular plasma membrane and the capsid is transported to the nucleus (A). The DNA is released into the nucleus where viral gene expression takes place in three stages initiated by VP16, an HSV transactivator protein (B). The first round of gene expression yields alpha proteins which induce the expression of beta genes. Beta proteins then induce the viral DNA replication, thus allowing the expression of gamma genes (C). Latent infection by HSV-1 (2.) is established in sensory neurons in the trigeminal ganglia where the virus enters the nucleus of the neuron after being transported through the connecting axon from the initial site of infection in the epithelial cells (G). Viral DNA remains as a circularized DNA molecule in the nucleus and does not integrate into the host genome (H). The production of viral proteins and infective particles is repressed and only LAT-RNA, and the microRNAs encoded in its sequence, are continuously expressed in the cell, preventing it from undergoing apoptosis (H). (Marcocci et al. 2020)*

Alpha proteins are mainly involved in regulating viral replication (Widener and Whitley 2014). Beta proteins are responsible for DNA synthesis and packaging (Widener and Whitley 2014), while gamma proteins mostly consist of structural virion proteins such as capsid, tegument and envelope proteins (Hukkanen and Seppänen 2020). Many virulence factors are also expressed

under gamma regulatory conditions. The viral capsid is formed in the nucleus and virions gain their first envelope from the inner nuclear membrane. However, the initial envelope is most likely lost once HSV particles leave the nucleus. Virions then make their way to the cell surface and gain their final envelope during this process while utilizing cellular membranes and vesicles to exit the cell. (Mettenleiter et al. 2013; Hukkanen and Seppänen 2020.)

As mentioned before, a distinguishing factor of HSV infections is latency which is established in sensory neurons when the virus is transported from the epithelial cells in the initial site of infection through the connecting nerve axon into the nucleus of the neuron (Whitley and Roizman 2001; Hukkanen and Seppänen 2020). The virus does not integrate into the host genome but remains as an episomal DNA molecule in the nucleus instead. No infective viral particles or proteins are produced in the nerve cell and only the latency associated RNA (LAT-RNA) is continuously expressed. (Roizman and Whitley 2013.) The LAT-RNA keeps the host neuronal cell alive by preventing it from undergoing apoptosis (Perng et al. 2000). Numerous microRNAs (miRNA) are coded in the LAT genomic region, both in HSV-1 and HSV-2 (Umbach et al. 2008; Jurak et al. 2010). The LAT-RNA can also be activated by different stimuli such as UV-light, stress or other infections which causes viral replication to reactivate. Typically, this takes place in one or a few neuronal cells at a time and results in the eventual destruction of the neuronal cell. After this the newly synthesized virions travel back through the axon to the initial site of infection where they cause a recurrent infection in the epithelium. (Roizman and Whitley 2013; Hukkanen and Seppänen 2020.)

1.1.4 Host responses to HSV infection

The immune system of vertebrates consists of two components; innate immunity which an individual is born with and adaptive immunity which develops via confrontations with pathogens. Host cell responses are shut off after HSV infects a cell. As a result, many host cell proteins are degraded, some of their gene expression is repressed and apoptosis is prevented (Paavilainen 2017). HSV has a remarkable ability to affect and evade both of these systems as it has over 20 ways to interfere with and hide from host immune responses. It can for example prevent the identification by and functions of many immune cells including macrophages, dendritic cells, cytotoxic and CD4+ T cells, as well as natural killer (NK) and Lymphokine-activated killer (LAK) cells.

Additionally, HSV can prevent IFN responses, apoptosis and necroptosis and evade Toll like receptors (TLRs). (Hukkanen and Seppänen 2020.)

HSV achieves T cell evasion by inhibiting Major histocompatibility complex (MHC) class I presentation of the virus. More specifically, HSV's Infected cell protein 47 (ICP47) prevents antigen presentation to CD8+ T cells by preventing peptide loading to MHC. (Früh et al. 1995; Hill et al. 1995.) While T cell evasion leaves HSV susceptible to NK cells at least HSV-2 has the ability to interfere with their function (Bellner et al. 2005). HSV also interferes with host responses through several of its glycoproteins. HSV's glycoprotein C (gC) for example blocks the complement system component C3b which in turn inhibits the activation of the alternative and classical pathways of the complement system (Fries et al. 1986). In addition, gC together with glycoprotein E (gE) have the ability to shelter other HSV glycoproteins blocking the virus's recognition by antibodies (Hook et al. 2008). Lastly gE and glycoprotein I (gI) of HSV inhibit antibody mediated adaptive immune responses by binding together and forming an Fc receptor that further binds to the Fc part of immunoglobulin G (IgG) (Johnson et al. 1988).

1.2 RNAi based therapies

RNA interference (RNAi) is a post transcriptional gene silencing mechanism first discovered in *Caenorhabditis elegans* in 1998 by Andrew Fire and Craig Mello. This discovery established that non-coding RNAs play an essential role in regulating gene expression in multicellular organisms (Fire et al. 1998). Later it was reported that RNAi could be triggered in mammalian cells by short 21-22 nucleotide (nt) long double stranded RNA (dsRNA) molecules without an induction of non-specific IFN responses (Elbashir et al. 2001). Short interfering RNAs or siRNAs have become an essential part of biological research because of their ability to silence any gene based on sequence complementarity. More importantly the discovery of siRNAs allows for the potential to develop versatile drugs that can target proteins previously considered undruggable. The sequence specific nature of RNAi based drugs also comes with the benefit that siRNAs can be retargeted without changing the overall formulation of the drug (Setten et al. 2019).

1.2.1 RNAi pathway through the lens of microRNA function

In mammalian cells the RNAi pathway starts in the nucleus with the transcription of primary microRNAs (pri-miRNAs) that are cleaved by a protein complex called Microprocessor (Fig. 4). Microprocessor comprises proteins Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) and the cleavage produces short hairpin RNAs (shRNAs) termed pre-miRNAs. (Lee et al. 2002; Gregory et al. 2004.) These RNAs are bound by Exportin 5 and transported to the cytoplasm (Yi et al. 2003) where Dicer and TAR RNA binding protein (TRBP) engage with it allowing for the cleavage of the pre-miRNA terminal loop by Dicer (Chendrimada et al. 2005). This in turn induces the formation of RLC (the RNA-induced silencing complex (RISC) -loading complex) with one of four Argonaute (Ago1-Ago4) proteins (Meister et al. 2004; Maniataki and Mourelatos 2005). RLC loads the antisense strand, also called the guide strand, of the miRNA onto the Argonaute protein while the sense or passenger strand is subsequently discarded and degraded (Matranga et al. 2005). With this the mature RISC, which comprises an Argonaute protein and the guide RNA, is ready to bind to messenger RNAs (mRNAs) that are complementary to its guide strand and inhibit their translation with a key mediator protein called GW182 (Eulalio et al. 2008; Takimoto et al. 2009). RISC achieves this by inducing sequestration of mRNAs into cytoplasmic P or GW bodies or by otherwise promoting mRNA degradation (Liu, Rivas, et al. 2005; Liu, Valencia-Sanchez, et al. 2005; Gibbings et al. 2009). Ago2 bears particular interest in the RNAi pathway as it is able to intrinsically cleave mRNAs (Liu et al. 2004; Meister et al. 2004). Additionally, an mRNA need not be fully complementary to the guide strand as it is enough that complementarity spans the seed region (bases 2 – 8 from the 5’ end) of the guide strand (Lewis et al. 2005).

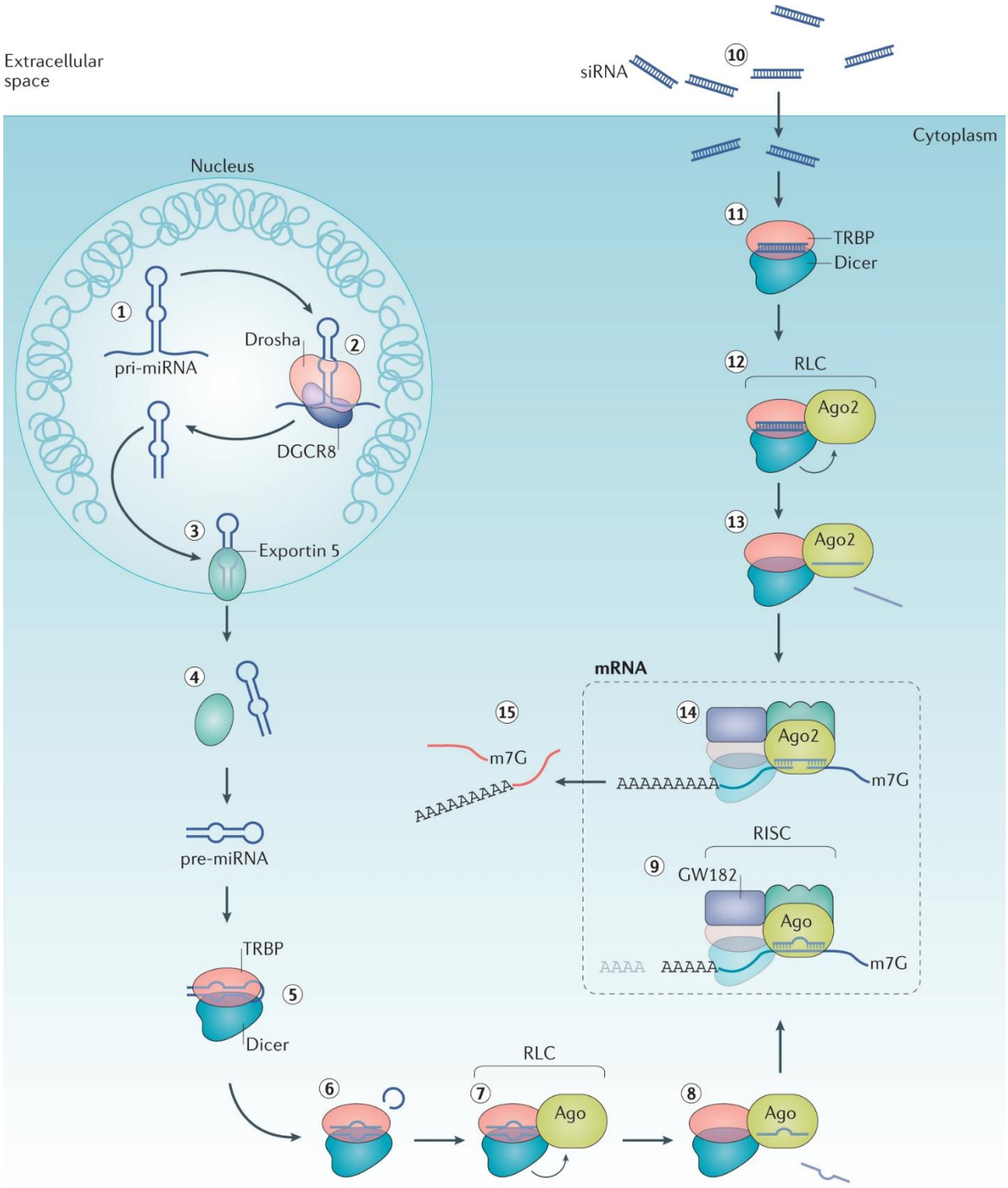

Figure 4. The RNAi pathway of miRNAs and siRNAs. *In the nucleus of mammalian cells pri-miRNAs (primary microRNAs) are transcribed and subsequently cleaved by a protein complex called Microprocessor which comprises proteins Drosha and DGCR8. Cleavage produces sh-RNAs (short hairpin RNAs), termed pre-miRNAs, which are transported to the cytoplasm by Exportin 5. Here proteins TRBP and Dicer associate with the pre-miRNA which allows Dicer to cleave the terminal loop of the RNA. After this RLC (RISC loading complex) is formed with one of four Ago proteins*

(Ago1-Ago4). RLC loads the antisense or the guide strand of the miRNA onto the Ago protein and the sense or passenger strand is discarded and degraded. The mature RISC, which comprises the Ago protein and the silencing guide strand, is formed following this event and together they bind to mRNAs complementary to the guide RNA of RISC. Along with a key mediator protein GW182 the transcription of bound mRNAs is prevented and they are sequestered to cytoplasmic P or GW bodies or are otherwise degraded. Exogenous siRNAs which enter cells through endocytosis and undergo endosomal escape are also able to induce RNAi. Similarly to miRNAs, siRNAs associate with TRBP and Dicer which induces the formation of RLC with the Ago2 protein. The guide strand is selected and loaded onto Ago2 and the mature RISC binds mRNAs complementary to its guide RNA. Ago2 which harbours intrinsic slicer activity cleaves the mRNA thus silencing it and preventing its translation into protein. m7G denotes 7-methylguanosine. (Setten et al. 2019)

1.2.2 RNAi triggers

RNAi can be triggered by synthetic small interfering RNAs that are usually designed to target a single mRNA (Elbashir et al. 2001). After entering the cell through endocytosis and undergoing endosomal escape, the synthetic siRNAs enter the silencing pathway by directly interacting with Dicer and TRBP (Fig. 4) (Haase et al. 2005; Wittrup et al. 2015). RLC is formed following this interaction which then induces guide strand selection, mature RISC production and subsequent gene silencing (Snead et al. 2013).

Synthetic RNAi triggers, which are generally perfectly base paired dsRNAs or short hairpin RNAs (shRNAs), may have a variety of structural motifs (Setten et al. 2019). Their lengths vary between 15 and 30 bp, since RNA triggers shorter than 15 bps cannot engage with the RNAi machinery while ones over 30 bps may induce nonspecific cytotoxic effects (Kim et al. 2005). There are also functional differences between siRNAs varying in length. siRNAs of 25/27 nt or 25 bps act as substrates for Dicer (DsiRNAs) and have better silencing efficacy and a higher chance of antisense strand selection than 21/21 nt or 19 bp siRNAs which are non-Dicer substrates (Snead et al. 2013). The latter siRNAs have better metabolic stability when not encapsulated in nanoparticles because their nature as non-Dicer substrate allows for the entirety of the siRNA and its 5' terminal phosphates to be chemically modified to achieve this (Parmar et al. 2016). All in all, different structural motifs serve different purposes. Some motifs are more efficient for RNAi processing,

some aid with guide strand selection between antisense and sense strands and some are better equipped to handle chemical modifications (Setten et al. 2019).

RNAi triggers may also include chemical modifications that are widely used in the development of RNAi drugs and serve two main purposes. Firstly, their inclusion helps RNAi triggers avoid activating immune responses and secondly, they prevent RNAi triggers from being degraded by endogenous exo- and endonucleases (Robbins et al. 2007; Bramsen et al. 2009). Chemical modifications may serve other additional purposes such as enhancing correct guide strand selection during RISC loading, reducing off-target effects by improved sequence selectivity and aiding in delivery to target sites by modifying the chemical and physical properties of the RNAi trigger (Bramsen et al. 2009; Janas et al. 2018; Setten et al. 2019). Some common chemical modifications to the backbone of the RNAi trigger include the incorporation of 2'-O-methyl, 2'-fluoro (Levanova et al 2020) and 2'-O-(2-methoxyethyl) groups (Khvorova and Watts 2017). These modifications improve nuclease resistance and reduce the induction of immune responses and off target effects (Bramsen et al. 2009; Ge et al. 2010; Khvorova and Watts 2017; Levanova et al. 2020).

1.2.3 Delivery of RNAi triggers

One of the biggest challenges of RNAi based therapies pertains the effective delivery of RNAi triggers to their target cells. These challenges are posed by the large size and negative charge of dsRNAs that cannot be addressed with chemical modifications alone and that affect systemic circulation, tissue penetration, cellular uptake and endosomal escape (Wittrup and Lieberman 2015; Setten et al. 2019). The first delivery issue is faced during administration to the bloodstream where naked RNAs are prone to nuclease degradation and can trigger innate immune responses (Layzer et al. 2004; Sioud 2005). RNAi triggers may also interact with serum components which can hinder their delivery to target sites (Malek et al. 2009). However, serum components such as lipoproteins can also be exploited by delivery systems to aid cellular uptake (Wolfrum et al. 2007).

The next challenge is encountered when naked RNAs enter the kidneys where they are too small in size to prevent their filtration from the circulation into urine (Huang et al. 2011). Thus, delivery systems must counter RNA clearance from the body while also avoiding being degraded in kidneys themselves (Zuckerman et al. 2012). Once RNAs have cleared their way through the kidneys they must exit the bloodstream and enter their target tissues through the endothelium. Delivery

becomes more difficult when tissue endothelia is continuous because lack of permeability leaves few openings for RNAs and their possible delivery vehicles to diffuse from the bloodstream into target cells. (Kanasty et al. 2013.)

The last two challenges are faced during cellular uptake and loading onto the RISC machinery. RNA molecules are too large in size to cross cell membranes and cellular uptake usually occurs through endocytosis (Wittrup and Lieberman 2015). This process can be assisted with targeting ligands that aid RNA internalization by interacting with cell surface receptors (Yu et al. 2009). If RNAs enter cells through endocytosis they must be released by the endosome into the cytoplasm. Endosomal escape is inefficient by itself, and endosome membrane disruption must be enhanced for higher levels of RNAs to be released into the cytoplasm (Wittrup and Lieberman 2015). In the cytosol RISC guide strand selection and loading happens to the RNA strand whose 5' end is most unstably hybridized. Because this end is crucial for RISC loading, conjugate delivery material attachment to the 5' end of the antisense strand is usually avoided. (Schwarz et al. 2003.) Other chemical modifications to the RNAi trigger backbone must also be carefully chosen to prevent mistakes in antisense strand selection as the guide strand during RISC loading (Kanasty et al. 2013).

Taking into account all of the delivery issues mentioned above, there has been a dire need to develop chemical excipients to overcome these challenges such as nanoparticles, lipid nanoparticles (LNPs) and nucleic acid nanostructures as well as polymers, dendrimers, exosomes and GalNAc- conjugated melittin- like peptides (NAGMLPs). For specific targeting purposes siRNAs have been attached to ligands such as aptamers, antibodies, peptides and small molecules like GalNAcs. (Setten et al. 2019.)

1.2.4 RNAi based therapies against diseases

The convenience of RNAi based therapeutics lies in the fact that, in theory, any disease-causing gene in any cell type can be silenced by RNAi triggers because all cells contain the necessary RNAi machinery. As such, potential drug candidates are much more numerous than enzymes and receptors targeted by small molecule drugs. Careful target selection in RNAi based therapies is important because knockdown of genes with non-redundant functions may lead to anticipated or unanticipated direct toxicities. (Wittrup and Lieberman 2015.) However direct toxicities do not necessarily occur if the target gene has a null or hypomorphic mutation which causes no symptoms or is even protective for the patient (Cohen et al. 2006). Conversely in certain diseases

it might be possible and even beneficial to silence genes that would otherwise be needed in a healthy individual. While silencing such genes is undoubtedly risky, exploring this research avenue might still be worthwhile. (Tabernero et al. 2013; Wittrup and Lieberman 2015.)

As mentioned before, delivery is one of the biggest issues hindering the development of RNAi based therapies. This is because delivery vehicles must work in a clinical setting. Currently, hepatocytes and the liver are the most accessible targets and delivery to them is no longer seen as a problem (Akinc et al. 2010; Shi et al. 2011; Wittrup and Lieberman 2015). Following these target sites are the skin, mucosa and eyes which can be accessed through topical application or injection. The most suitable delivery method for most of these targets seems to be cholesterol-conjugation (Wittrup and Lieberman 2015).

RNAi based therapies have been in clinical development for a wide range of diseases including viral infections (discussed in the next chapter), cancer as well as cardiovascular, ocular and rare genetically inherited diseases (Setten et al. 2019). An important example of a successful RNAi drug used in clinical settings is patisiran (Onpattro, Alnylam Pharmaceuticals). This siRNA drug is an FDA approved medication used for the treatment of transthyretin (TTR) amyloidosis, a rare inherited neurodegenerative disease. This life-threatening disease is caused by deposition of TTR amyloids in the peripheral nervous system and organs such as the heart and gastrointestinal tract. (Adams et al. 2017.) TTR proteins are mainly produced in the liver and can have over 120 hereditary ATTR (hATTR) disease causing mutations (Holmgren et al. 2008; Rowczenio et al. 2014).

Until the development of patisiran, treatment options for hATTR were limited. Small molecules were used to stabilize TTR tetramers in their native conformation but this only worked to slow disease progression and better treatment options were desperately needed. (Ando et al. 2013; Adams et al. 2018.) Patisiran was developed to silence both wild type and mutated *TTR* genes in hepatocytes by using a siRNA that targets the 3' untranslated region in the *TTR* mRNAs (Coelho et al. 2013). The target site was chosen because it is believed to be more homologous across the patient population. By targeting this site a wide range of *TTR* mRNA variants could be silenced. (Butler et al. 2016.) Contrary to many siRNA drugs undergoing clinical development patisiran's formulation does not rely on extensive chemical modifications, metabolic stabilization or a targeting ligand. Instead, the siRNA is encapsulated into a lipid nanoparticle that has been designed for efficient uptake by hepatocytes. (Coelho et al. 2013; Adams et al. 2018.) While patisiran as a drug brings hATTR patients new hope that disease progression can be halted, the safety and efficacy of the drug's use over 18 month long periods is still unclear (Setten et al. 2019).

1.2.5 RNAi based therapies against viral infections

RNAi, while a means of gene regulations also acts as a natural antiviral defence system used by both plants and animals such as insects and nematodes (Li and Ding 2006; Zhang and Qu 2014; Chin et al. 2017). When used for antiviral defence the siRNA pathway is utilized to process viral RNA into virus derived small RNAs (vsRNAs). These RNAs are naturally complementary and specific to the viruses they originate from and can even be amplified by the host to enhance RNAi mediated antiviral defence. (Chin et al. 2017.) Whether the mammalian siRNA pathway also acts as an antiviral defence mechanism is still under controversy and requires further investigation (Seo et al. 2013; Gantier 2014).

In theory the use of RNAi as an antiviral treatment is ideal because viruses replicate in high numbers, they are dependent on a limited set of genes and the target genes are usually nonhomologous to human genes. RNAi based antiviral therapies also have an advantage in development time because it would be faster to develop a siRNA against a newly isolated virus than it would be to develop a small inhibitor drug. Additionally, even if a virus develops resistance to a siRNA drug or otherwise mutates, the sequence of the siRNA can be readily altered to match the mutated virus. (Blake et al. 2012.)

Generally speaking, RNAi is targeted against genes that are essential for viral infection, replication and overall survival. Target genes need not be within the virus itself and instead RNAi can be used to silence host genes that are essential for the virus but not the host itself. Such targets include surface receptor encoding genes that the virus needs for cell entry or genes related to intracellular viral replication. While tampering with regular host gene function carries its own risks, targeting host genes instead of viral genes is may be an attractive option when the virus mutation rate is high. (Blake et al. 2012.)

RNAi targeting occurs in a sequence-specific manner and as a result of its high specificity off-target effects are limited. This makes siRNA drugs generally well tolerated, compatible with other antiviral treatments and potentially well suited for long-term and prophylactic therapies. (Alvarez et al. 2009; Zamora et al. 2011; Chin et al. 2017.) Because it is RNA that is cleaved during the RNAi process, RNA viruses whose replication occurs in the cytoplasm are especially susceptible to gene silencing by this mechanism (Stein et al. 2011; Suhy et al. 2012). Since one siRNA molecule is able to silence multiple target mRNAs, off-target effects can be further reduced by using siRNA drugs in

low concentrations. Thus, siRNA drugs need not be administered in a proportionate amount to the virus for treatments to be effective. (Suhy et al. 2012; Chin et al. 2017.) It is important to note that siRNA drugs by themselves can trigger an immune response through the IFN pathway and this effect is usually undesirable (Alvarez et al. 2009). However, the IFN response can also aid in viral clearance and studies have been conducted to determine whether the immune response triggering trait of RNAi drugs could be exploited in a combination therapy that elicits viral clearance both through the RNAi pathway and the IFN pathway (Ebert et al. 2011; Chen et al. 2013).

Finally, RNAi can not only be utilized as a treatment itself but can be used as a screening method to search for potential new genes whose function is essential for the virus. These essential viral genes can then act as potential targets for antiviral drug development. In fact, siRNA and shRNA based libraries can be used as genome wide screening tools to study the biology of viruses as a whole. (Blake et al. 2012; Snijder et al. 2012.) The principle of screening is simple and involves the transfection of seeded cells with siRNAs or shRNAs and the subsequent infection of cells with the virus under study. If cells show no signs of infection, then a gene vital for viral replication has been silenced. Typically, siRNAs are transfected into cells using a liposome based delivery system while shRNAs transfection happens through viral or other vectors. (Blake et al. 2012.)

RNAi has been used to inhibit several viruses in both cell culture, animal models and clinical trials. Some of these viruses include HSV, respiratory syncytial virus (RSV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), HIV, influenza and Ebola (Watanabe et al. 2006; Zhou et al. 2007; Sima et al. 2008; DeVincenzo et al. 2010; DiGiusto et al. 2010; Dunning et al. 2016; Paavilainen et al. 2017; Yuen et al. 2020).

1.3 Synthesis of antiviral siRNA swarms

Therapeutic siRNAs can be synthesized in three different ways: enzymatically, chemically or using expression vectors or cassettes. Chemical siRNA synthesis starts with the production of a single stranded RNA (ssRNA) through automated solid-phase synthesis, which yields ribonucleoside phosphoramitides. Thereafter, RNA duplexes are formed by hybridization of the produced ssRNAs. Each produced ssRNA is tested by matrix-assisted laser desorption-ionization mass spectrometry before siRNA duplexes are formed. siRNAs themselves are also tested for proper annealing by

running them through non-denaturing gel or capillary electrophoresis. (Levanova and Poranen 2018.)

Expression cassettes can also be utilized for siRNA production (Brummelkamp 2002). These cassettes are delivered into cells via plasmids or viral vectors (Levanova and Poranen 2018) and incorporate a polymerase III promoter followed by an shRNA sequence in its DNA form and a transcription stop signal (Brummelkamp 2002). Once inside the cell, the shRNA is transcribed from the cassette and cleaved by Dicer to produce the desired siRNAs which can then associate with RISC and induce silencing of target genes (Brummelkamp 2002).

In enzymatic siRNA production, synthesis takes place *in vitro* from a vector that contains the DNA template sequence for the desired siRNA and a T7 polymerase promoter (Levanova and Poranen 2018). The T7 DNA-dependent RNA polymerase (DdRp) transcribes both sense and antisense strands of these DNA sequences. Thereafter the produced ssRNAs are annealed. (Sohail 2003.) The T7 DdRp adds a leader sequence to the 5’ end and possibly random nucleotides to the 3’ end of the ssRNA molecules (Sohail 2003) as well as a triphosphate also to the 5’ end (Kim et al. 2004). However, the leader sequence can be cleaved off by deoxyribozyme (Sohail 2003) and IFN induction of the 5’ triphosphate can be nullified by treating ssRNAs with alkaline phosphatase (Kim et al. 2004). Conversely, the random nucleotides at the 3’ end do not have harmful effects on the safety and efficacy of produced siRNAs (Sohail 2003).

Each mentioned approach comes with its advantages and disadvantages. Chemical synthesis for example is still relatively costly despite synthesis costs per nucleotide decreasing vastly during the past years. The advantage of chemical synthesis is that it produces defined sequences of ultrapure siRNAs in large quantities which are suitable for use in clinical trials, for example. Expression vectors on the other hand are more suited for long term siRNA production. Traditional enzymatic siRNA synthesis suffers from limited siRNA yield due to inefficient hybridization that produces inactive siRNAs. This is especially true in the case of long ssRNAs which form tertiary structures that prevent the annealing of complementary strands into an RNA duplex. However, enzymatic siRNA synthesis has the advantage of rapid, low-cost production and can be used as a screening tool for finding efficacious siRNA sequences. (Levanova and Poranen 2018.)

1.3.1 siRNA swarm design and enzymatic synthesis

Instead of using one single siRNA to silence a gene of interest, a mixture of siRNAs with differing sequences that target the same gene can be used for silencing purposes. These pools or swarms of enzymatically synthesized siRNAs have several advantages over single site siRNAs, especially when used for antiviral purposes. Enzymatic synthesis is fast and robust, which means that in the case of a sudden virus outbreak antiviral siRNA swarms can be produced rapidly. Because siRNA swarms cover a larger area of the viral genome, they are more protective than short single site siRNAs. (Levanova and Poranen 2018.) Swarms can also be used against viruses with high mutation rates, as the mixture of siRNAs will more effectively inhibit the virus while preventing the generation of new escape mutants (Gitlin et al. 2005). Lastly, siRNA swarms have the potential to be more highly tolerated with reduced off target effects, because each individual siRNA in the mixture is present at very low concentrations (Aalto et al. 2007; Romanovskaya et al. 2012; Paavilainen et al. 2016, 2017).

As mentioned before, siRNAs have been used to target several viruses. Before antiviral siRNA production takes place, the sequences of these siRNAs must be carefully designed and selected in silico. Typically, siRNAs are targeted against essential genes in highly conserved areas in the viral genome. This is done to achieve maximal antiviral efficacy while reducing off-target effects. Sequences that match closely with human or model animal transcripts are excluded with the use of specific filters. (Levanova and Poranen 2018.) Additional structure and sequence-based algorithms are also utilized to select siRNA sequences with the highest functionality (Reynolds et al. 2004; Levanova and Poranen 2018). Generally speaking, enzymatic siRNA swarm production follows the same in silico steps as single site siRNAs synthesis except for the final step of using of algorithms to find the most highly functional sequences. This step is not required in swarm production because swarms cover a much larger highly conserved area in the genome. (Levanova and Poranen 2018.)

Following in silico sequence design, the antiviral siRNA swarms complementary to the chosen target sequence can be produced. A single tube enzymatic synthesis platform has been developed by Aalto et al. (2007) where dsRNA production starts with the cloning of the target DNA template into a suitable vector under a T7 polymerase promoter (Fig 5.). The T7 DdRp then transcribes the DNA template into ssRNAs, which bacteriophage Phi6 RNA-dependent RNA polymerase (RdRp) uses for the production of the complementary strand by synthesizing the minus strand starting

from the 3' end of the original ssRNA plus strand. The use of Phi6 RdRp circumvents the inefficient ssRNA annealing step of traditional enzymatic siRNA synthesis which requires heating at a high temperature for the denaturing of secondary and tertiary structures. (Aalto et al. 2007.) Additionally, the enzyme lacks template specificity which means that it can also be used to produce dsRNA molecules of any length (Makeyev 2000). The final steps of siRNA swarm production involve the use of a recombinant eukaryotic Dicer enzyme to digest the long dsRNA molecules into smaller functional siRNAs that are specific to their target (Romanovskaya et al. 2012). These siRNAs are then purified with anion exchange chromatography followed by desalting which yields highly pure and safe to use pools of siRNAs (Romanovskaya et al. 2013).

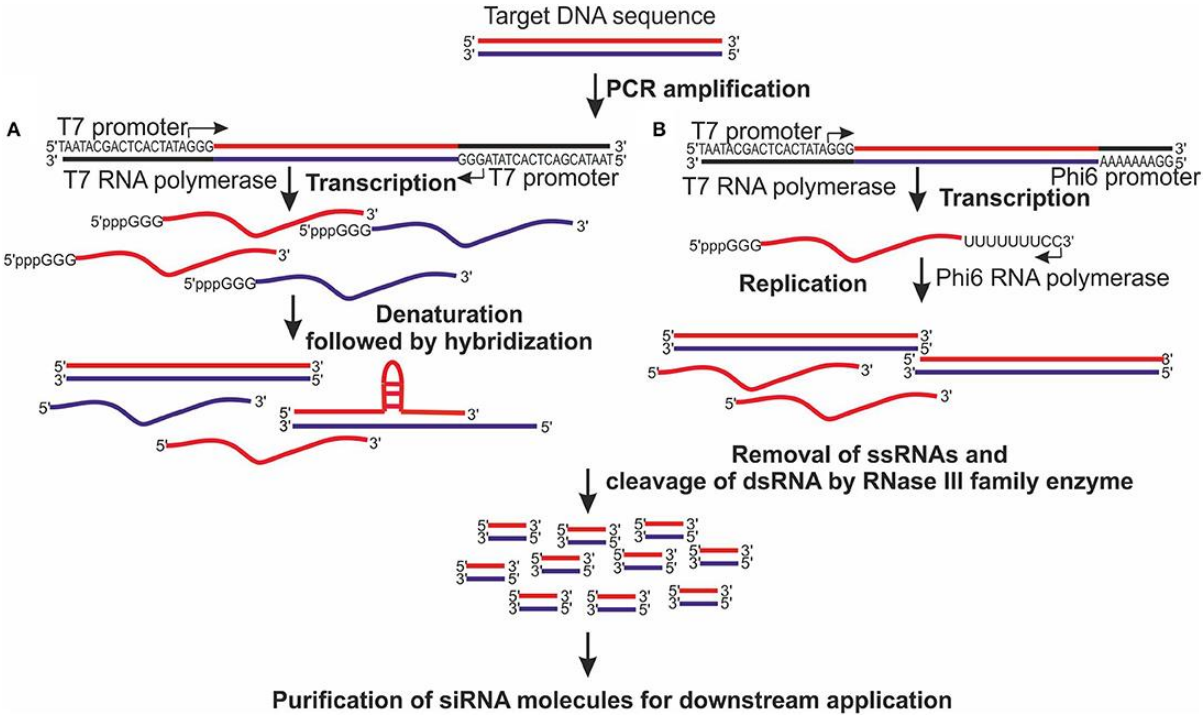

Figure 5. Steps of the enzymatic production of siRNA swarms. *Pools or swarms of siRNAs can be synthesized by PCR production from DNA templates containing the target site for silencing. A) One way to do this is with use of forward and reverse primers that contain the promoter sequence for the T7 DdRp at their 5' end. The T7 polymerase then transcribes the DNA templates into ssRNA molecules which require heating at a high temperature for the denaturing of secondary and tertiary structures if proper annealing of ssRNAs into dsRNAs is desired. Even so denaturing branched structures and hairpins becomes increasingly difficult with long ssRNA molecules which decreases the yield of full-length, biologically functional dsRNAs. B) Alternatively, PCR production of DNA templates can be achieved by switching the promoter sequence of the reverse primer from*

a T7 DdRp sequence to a Phi6 RdRp sequence. By doing so Phi6 polymerase replicates the second strand of the ssRNA molecule that T7 polymerase has transcribed from the DNA template in a single tube reaction. In both approaches (A and B) the newly synthesized dsRNAs are purified, cleaved by an enzyme belonging to the RNAse III family such as Dicer and further processed depending on the downstream application. The second approach (B) however has the advantage of producing a higher yield of biologically functional, full-length dsRNA molecules due to the use of Phi6 polymerase which removes the annealing step of the first approach (A). (Levanova and Poranen 2018)

1.3.2 siRNA swarm targets in HSV

The siRNA swarms used in this study were specifically developed against the HSV-1 genes *UL27*, *UL29* and *UL54*. These genes are all essential for the survival of the virus, making them ideal targets for an RNAi based antiviral treatment, and they represent the three regulatory groups of HSV genes denoted alpha, beta and gamma. *UL54* codes for a multifunctioning protein and represents the alpha group. The beta group is represented by *UL29,* which codes for a protein that partakes in DNA replication. Finally, the gamma group is represented by *UL27,* which is a gene coding for glycoprotein B that participates in virion entry. Aside from being essential genes and representing different regulatory groups, the genomic regions of these genes contained long enough target sequences for siRNA swarm development. The target sites did not share homology between human or mouse transcriptomes but had the advantage of being highly homologous between HSV-1 and HSV-2, as HSV-2 also contains the genes *UL27*, *UL29* and *UL54*. (Romanovskaya et al. 2012; Paavilainen et al. 2016.)

2 Aim of the study

It has been estimated that in 2016 approximately 3,8 billion and 491,5 million people globally carried an infection by HSV-1 and HSV-2 respectively (James et al. 2020). The detrimental effects of HSV can be far-reaching as the virus causes life-long latent infections that can lead to further complications such as infections in the ocular tissue, the CNS and as well as generalized infections in neonates and immunocompromised individuals (Hukkanen and Seppänen 2020). Additionally, HSV-2 infections increase HIV transmission which causes the spread of acquired immunodeficiency syndrome (AIDS), a severely harmful disease itself (Looker et al. 2017). While acyclovir is a fairly effective and commonly used drug for the treatment of HSV infections, ACV resistance ranges from 3.4% to 10% in immunocompromised patients (the prevalence in immunocompetent patients is 0.1 – 0.6%) (Christophers et al. 1998; Piret and Boivin 2011). According to some surveys resistance in immunocompromised individuals could be even higher (Langston et al. 2002; Morfin et al. 2004). All these facts taken together indicate that it is imperative that new drugs and treatments are developed to combat HSV infections worldwide.

Previously Paavilainen et al. (2012; 2016) have developed enzymatically synthesized siRNA swarms targeting the essential viral HSV genes *UL27*, *UL29* and *UL54*. The swarms were designed against the HSV-1 prototype virus strain 17+. All swarms were shown to have an inhibitory effect on HSV-1, with the *UL29* targeting swarm having the highest antiviral efficacy in both *in vitro* and *in vivo* studies with minimal adverse side effects (Paavilainen et al. 2016, 2017). The UL29 swarm was thus proven to be the best candidate for further drug development. Very few studies have been conducted on the efficacy of siRNA swarms against HSV-2, even though both HSV viruses contain the three highly homologous genes *UL27*, *UL29* and *UL54*. It is undeniable that an siRNA drug which can effectively inhibit both HSV-1 and HSV-2 would be an ideal drug candidate, especially for patients suffering from acyclovir resistance.

Because previous research has centered around HSV-1, my thesis will focus on evaluating the antiviral effects of the previously described *UL27*, *UL29* and *UL54* gene targeting siRNA swarms against clinical strains of HSV-2. The studies begin with the use of BLAST and MSA tools for *in silico* analyses of the sequence identity and conservation level of HSV-2 gene target sites of the siRNA swarms compared to corresponding sites in HSV-1 strain 17+. The analyses include seven fully sequenced clinical isolates, three reference strains and five clinical HSV-2 isolates, which I cultured and arranged the partial sequencing of. The results of the sequence identity analyses are used to

predict the results of the antiviral experiments. After this Vero cell culture is used for the propagation and titration of HSV-2 strains, which will determine the virus production of viral stocks in plaque forming units (pfu). The actual antiviral experiments are carried out on microtiter well-plates in two representative cell lines, HaCaT and mink lung cells (MLC) where cells will be transfected with antiviral siRNA swarms or controls and later infected with an HSV-2 strain under study. A commercially available siRNA that has been specifically designed by Palliser et al. (2006) to combat HSV-2 infections is used as a positive control in the study. The effects of siRNAs on viral gene expression and replication are determined by 1) calculating the pfu of the produced virus after transfection and by 2) isolating DNA from the supernatant of treated cells and performing quantitative PCR analysis for the HSV-2 *gD* gene, which will determine the genome copy number.

3 Materials and methods

3.1 Cell lines and viruses

Antiviral studies were conducted in two cell lines, human epithelial keratinocyte (HaCaT) cells and mink lung cells (MLC). Cells were maintained in DMEM with 2 mM L-glutamine and 7% heat inactivated foetal bovine serum (FBS). Virus propagation and plaque titration was carried out in African green monkey (Vero) cells which were maintained in DMEM supplemented with 2% FBS during infection steps or 7% FBS during maintenance. All cell lines were incubated at +37 °C in 5% CO_2.

One reference strain of herpes simplex virus type 2 (Human herpesvirus 2, genus Simplexvirus, family Herpesviridae), denoted HSV-2 G, and nine clinical strains were used in the study. Clinical strains were obtained from herpes lesions and collected from anonymous archival diagnostic samples. The viral isolates were designated as #10, #24, #50, #72, #84, H1226, H12227, H1229 and H12211. To prepare viral stocks from clinical samples, viruses were propagated on Vero cells after which 9% sterile milk in water was added to infected cells. Cells were lysed by freezing and thawing. Viral stocks used in the study were prepared from the second passage on Vero cells by pelleting the infected cells to clarify the supernatants. Titers of viral stocks were determined by plaque titration in Vero cells on 48- or 96-well microtiter plates. Cells were infected with a virus under study and maintained in DMEM supplemented with 7% FBS and 0.04 mg/ml human immunoglobulin G. Three days post infection cells were fixed with methanol for 10 min and stained with 0.1% crystal violet in PBS, after which plaque forming units (pfu) from infected cells could be counted.

3.2 Enzymatically synthesized anti-HSV siRNA swarms

Anti-HSV siRNA swarms were prepared from DNA template sequences covering HSV-1 genes *UL27*, *UL29* and *UL54* from the HSV-1 prototype strain (17+) (GenBank JN555585). Template sequences were located in nucleotides 54689–55207 for the *UL27* gene, 59301–59953 for the *UL29* gene and 113947–114715 for the *UL54* gene in the prototype strain. PCR was used to amplify DNA templates using the primers listed in Table 1. Bacteriophage T7 and Phi6 polymerases were employed to synthesize dsRNA molecules from the PCR products in our collaborative laboratory of

Dr. Minna Poranen, University of Helsinki. The dsRNA molecules were then digested by *Giardia intestinalis* Dicer endonuclease to yield canonical pools or swarms of 25-27 nt long siRNAs as described previously (Romanovskaya et al. 2012). Finally, siRNA swarms were purified as detailed by Romanovskaya et al. (2012) using anion-exchange chromatography on CIMac QA columns (BIA Separations, Slovenia) using the AKTApurifier FPLC system (GE Healthcare). A non-specific eGFP siRNA swarm described by Romanovskaya et al. (2012), which has no target in HSV, was also included in the studies as a control.

Table 1. List of primer sequences used in the study. *Forward/reverse and sense/antisense primers were used in the siRNA swarm production, gene sequencing, PCR and quantitative PCR steps of the study.*

Primer sequences used in siRNA swam production (HSV-1 prototype sequence)

UL27-T7 sense	TAA TAC GAC TCA CTA TAG GGC ACT TGG TCA TGG TGC AGAC
UL27-phi6 antisense	GGA AAA AAA GGT GAT CGA CAA GAT CAA CG
UL29-T7 forward	TAA TAC GAC TCA CTA TAG GGA TGA TGG CCG TAA GGG TGT
UL29-phi6 antisense	GGA AAA AAA CGC AAC TTT CGC AAT CAA T
UL54-T7 sense	TAA TAC GAC TCA CTA TAG GGC CGT CTC GTC CAG AAG ACC
UL54-phi6 antisense	GGA AAA AAA CGG CAA AAG TGC GAT AGA G
eGFP-T7 sense	TAA TAC GAC TCA CTA TAG GGA TGG TGA GCA AGG GCG AGG AG
eGFP-Phi6 antisense	GGA AAA AAA CTT GTA CAG CTC GTC CAT GCC G

Primer sequences used in sequencing

UL27 Forward	GAG GAG AAG CGG AAG GAG C
UL27 Reverse	CGC TAC TCC CAG TTT ATG GGG
UL29 Forward	CCT GCA CGC TGG GGG
UL29 Reverse	CAG TGC CAC GGG GTG TTC
UL54 Forward	GAG ACG GAG GGG CGG A
UL54 Reverse	GCA TGG CCT TGG CTG TCG

Primer sequences used in qPCR

HS2D-1U, forward	ACC CAC CGC ACC ACC ATA CTC
HS2D-4U, reverse	GCG ACT AGT GGT TCG CAA TGC A

3.3 Sequencing of target genes from clinical isolates

Vero cells were infected with HSV-2 clinical isolates with 0.1 pfu/cell. After the CPE (cytopathic effect) of infection reached 100%, cells were harvested and lysed with Lyse-O-Lot (150 mM NaCl, 10 mM Tris pH 7.6, 1.5 mM MgCl, 0.1% NP-40). The nuclei were then pelleted gently and beta-mercaptoethanol as well as 0.5 M EDTA pH 8 was added to the supernatant. Phenol-chloroform extraction and ethanol precipitation was used to isolate viral DNA. To sequence genes *UL27*, *UL29* and *UL54* from HSV-2 clinical isolates, PCR amplification was used to amplify the gene sequences using the primers listed in Table 1. Amplified DNA was run on a 0.8% agarose gel and the bands corresponding to the genes were excised and purified from the gel using GeneJet gel extraction kit (Thermo Fisher Scientific), following the manufacturer's protocol. Purified DNA was then sent to sequencing (LightRun, Eurofins Genomics) in sealed eppendorf tubes containing an equal volume of DNA and primers.

3.4 Transfection and infection

MLC and HaCaT cells were transfected on 96-well microtiter plates at 30 – 60% confluency with 10 pmol/well of enzymatically synthetized siRNA swarms, a single site siRNA, or water. Cells were first washed once with optiMEM transfection medium and transfected 1 h later using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's forward transfection protocol. 4 hours post transfection cells were washed twice with DMEM supplemented with 2% FBS after which the same type of medium was used to infect cells with the HSV-2 strain under study at a dose of 1000 pfu/well in a volume of 100 µl. 1 – 1.5 h post infection cells were washed twice and covered with 200 µl/well of DMEM supplemented with 7% FBS. Finally, 48 h post transfection, culture media was collected from cells to determine viral shedding by plaque titration and quantitative PCR analysis.

3.5 Quantitative PCR

DNA was extracted from supernatants of treated cells with the NucliSens EasyMag system following the manufacturer's instructions. To quantify the number of viral genomes in samples quantitative PCR (qPCR) was performed to amplify extracted DNA for the HSV-2 *gD* gene with the primers listed in Table 1. Reactions were carried out with the Maxima SYBR Green/ROX qPCR

Master Mix and the Rotor-Gene Q real-time instrument (Qiagen). The enzyme mixture was pipetted in a separate PCR clean room, where no PCR products or samples were handled. The total reaction volume was 20 µl containing 10 µl of SYBR Green/ROX qPCR master mix, 10 pmol of forward and reverse primers, 7 µl of water and 2 µl of DNA sample. The reaction was carried out as follows: initial denaturation at 95 °C for 10 min, 45 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 45 s, followed by melting curve generation from 72 to 95 °C for 90 s and then 5 s.

3.6 Computational analyses

Multiple sequence alignments were performed with BLAST (Basic Local Alignment Search Tool) to check the identity of antiviral siRNA swarms target sites in HSV-1 strain 17 + and the corresponding targets in several HSV-2 strains. Primer-BLAST was used to design primers for the sequencing of genes *UL27*, *UL29* and *UL54* from HSV-2 clinical strains. The CAP3 Sequence Assembly Program was utilized to assemble contigs from data gained from sequencing of genes *UL27*, *UL29* and *UL54*. IBM SPSS Statistics 25 software was used for statistical analyses. Two groups were compared at a time to calculate statistical significances using the non-parametric Mann-Whitney U-test. The statistical significance threshold was set to $p < 0.05$.

4 Results

4.1 Multiple sequence alignment analysis of target genes in HSV strains

Previously our group has described the *in vitro* and *in vivo* antiviral effects of siRNA swarms that target essential HSV-1 viral genes *UL27*, *UL29* and *UL54*. These swarms were produced using target sites of the HSV-1 strain 17+ (GenBank JN555585) as a reference. To predict whether these HSV-1 targeting siRNA swarms would silence the corresponding HSV-2 genes and thus inhibit the virus, the sequence identity of swarm target sites in HSV-1 and clinical HSV-2 strains were compared to each other. BLAST sequence alignments were run by aligning three HSV-2 reference strains (G, HG52, 333), seven fully sequenced (H1226, H1227, H1229, H12211, H12212, H1421 and H1526) and five partially sequenced (#10, #24, #50, #72 and #84) HSV-2 local clinical strains against the swarm target sites in HSV-1 strain 17+ for the genes *UL27*, *UL29* and *UL54*. Alignment results revealed that the target site for the UL29 siRNA swarm in HSV-2 strains were the most identical to the swarm target site in HSV-1 strain 17+ with 97-100% query cover and 95% identity (Table 2). The *UL27* gene target site had 100% query cover across all tested HSV-2 strains and resulted in 94% sequence identity. The least identical target site was in the *UL54* gene with 94 – 99% query cover and 75 – 79% sequence identity across different HSV-2 strains.

Table 2. BLAST sequence alignment of anti-HSV siRNA swarm target sites in HSV-1 and HSV-2. *The anti-HSV siRNA swarm target sites for the essential genes UL27, UL29 and UL54 in HSV-1 strain 17+ were aligned to corresponding target sites in several HSV-2 laboratory and clinical strains. Alignment results are represented as query cover (QC) and identity (%) for each gene.*

HSV-2 Strain	QC	*UL27*	QC	*UL29*	QC	*UL54*
G (US)	**100%**	**488/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**599/767 (78%)**
HG52 (Scotland)	**100%**	**488/519 (94%)**	**100%**	**619/653 (95%)**	**99%**	**601/767 (78%)**
333	**100%**	**489/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**601/767 (78%)**
H1226	**100%**	**488/519 (94%)**	**100%**	**620/653 (95%)**	**99%**	**601/767 (78%)**
H1227	**100%**	**489/519 (94%)**	**100%**	**619/653 (95%)**	**99%**	**600/767 (78%)**
H1229	**100%**	**488/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**601/767 (78%)**
H12211	**100%**	**489/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**601/767 (78%)**
H12212	**100%**	**488/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**601/767 (78%)**
H1421	**100%**	**488/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**601/767 (78%)**
H1526	**100%**	**488/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**600/767 (78%)**
#10	**100%**	**488/519 (94%)**	**98%**	**614/645 (95%)**	**94%**	**550/729 (75%)**
#24	**100%**	**488/519 (94%)**	**99%**	**620/652 (95%)**	**99%**	**601/767 (78%)**
#50	**100%**	**487/519 (94%)**	**100%**	**621/653 (95%)**	**99%**	**598/764 (78%)**
#72	**100%**	**487/519 (94%)**	**97%**	**605/639 (95%)**	**94%**	**574/725 (79%)**
#84	**100%**	**487/519 (94%)**	**100%**	**620/653 (95%)**	**99%**	**598/767 (78%)**

4.2 Antiviral efficacy in MLC and HaCaT cells

4.2.1. Virus titers in culture medium

To analyze the antiviral effects of HSV-1 targeting siRNA swarms on clinical strains of HSV-2, HaCaT cells and mink lung cells (MLC) were seeded on 96-well plates and transfected with 10 pmols of anti-HSV swarms or controls once cells reached 60% confluency. (Lower confluency allows for easier and more successful transfection.) Swarms were denoted UL27, UL29 and UL54 according to the genes they target. A previously published HSV-2 *UL29* gene targeting single site siRNA denoted UL29.2 was used as a reference. A non-specific siRNA swarm targeting *eGFP* as well as water transfection were used as controls. After transfections cells were infected with HSV-2 clinical strains H1226, H1227, H1229, H12211, #10, #24, #50, #72 or #84 with a dose of 1000 pfu/well. To quantify viral inhibition 48 hours post transfection, cell culture supernatants were collected and titered in Vero cell cultures to establish the titer of the shed virus as plaque forming units.

UL27, *UL29* and *UL54* gene targeting siRNA swarms all had varying degrees of an inhibitory effect on different HSV-2 strains in both HaCaT cells and MLC. The antiviral effects were more visible in MLC where plaque titration showed that in almost all cases infected, antiviral swarm treated cells shed significantly less virus than infected and water transfected or infected only cells (Fig. 6). Decreases in viral shedding ranged from 10-fold to even over a 100-fold. The antiviral effects of swarms are better visualized as inhibition rates by comparing infected, antiviral swarm treated cells to infected, mock-treated cells. In MLC *UL27*, *UL29* and *UL54* gene targeting swarms all reached from 70 to nearly 100% inhibition rates depending on the HSV-2 strain in question (Fig. 8).

In HaCaT cells the antiviral UL27, UL29 and UL54 siRNA swarms had a much less pronounced inhibitory effect on different HSV-2 strains. This was evidenced by the little to no difference in viral shedding of infected, swarm treated cells compared to infected, mock-treated and infected only cells (Fig. 7), as well as the relatively low inhibitory rates of antiviral swarms (Fig. 9). The UL27, UL29 and UL54 swarms that did have an inhibitory effect on some HSV-2 strains, namely #10 and #50 had inhibition rates ranging from 25 to 75%.

Interestingly the single site *UL29* gene targeting siRNA UL29.2 had a significant antiviral effect on some HSV-2 clinical isolates in HaCaT cells as well as in a few HSV-2 strains in MLC. The inhibition rate of the UL29.2 single site siRNA was much higher than those of the antiviral siRNA swarms in HaCaT cells, where the inhibition rate of UL29.2 ranged from 75 to 95% when the effect was significant (Fig. 9). In MLC however the antiviral UL27, UL29 and UL54 siRNA swarms reached higher inhibitory rates more consistently than the single site UL29.2 siRNA, whose inhibition rate ranged between 19% and 99% and more specifically 82 – 99% when the inhibitory effect was significant (Fig. 8).

While the non-specific eGFP siRNA swarm does not contain a target site in HSV-2 it did exhibit an inhibitory effect on several HSV-2 strains in both HaCaT cells and MLC. In HaCaT this antiviral effect could be seen in three strains, where the inhibition rate of the eGFP swarm ranged between 2 and 61% (Fig. 9). In MLC however it was surprising that the eGFP swarm could inhibit the virus almost at the same rate as the antiviral UL27, UL29 and UL54 siRNA swarms, as its inhibition rate was 82 – 99% (Fig. 8).

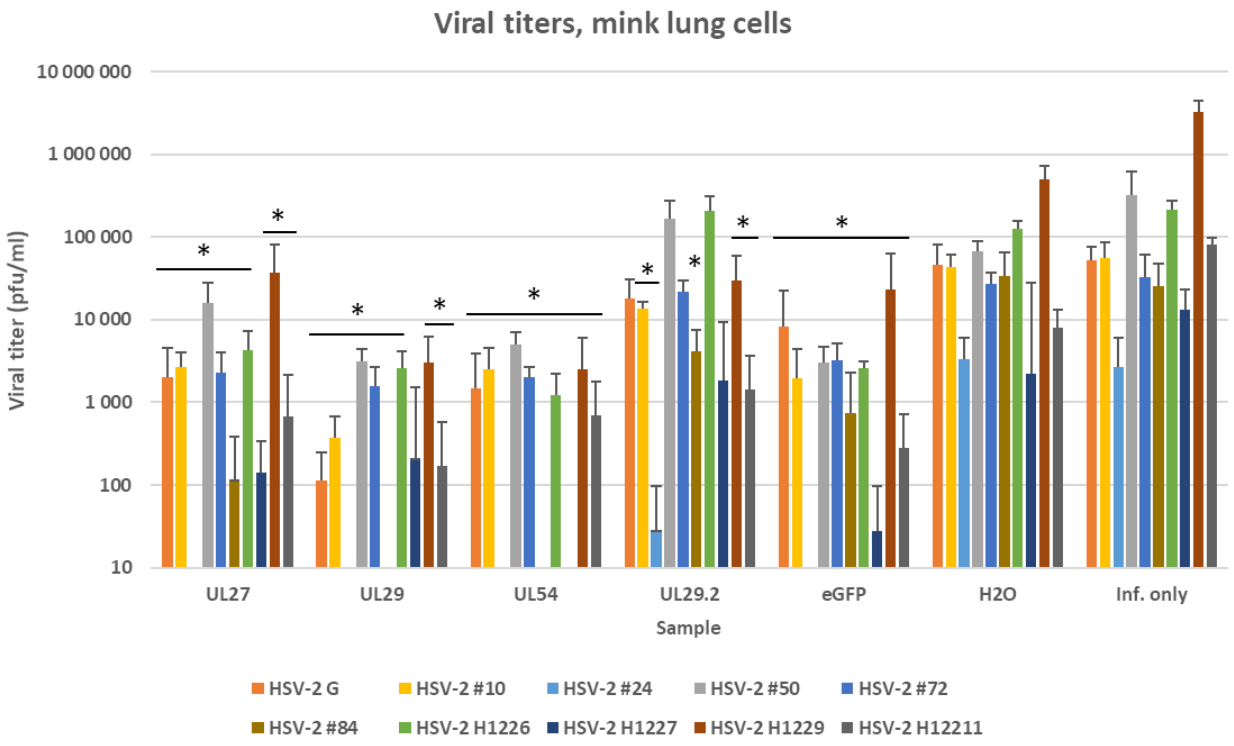

Figure 6.

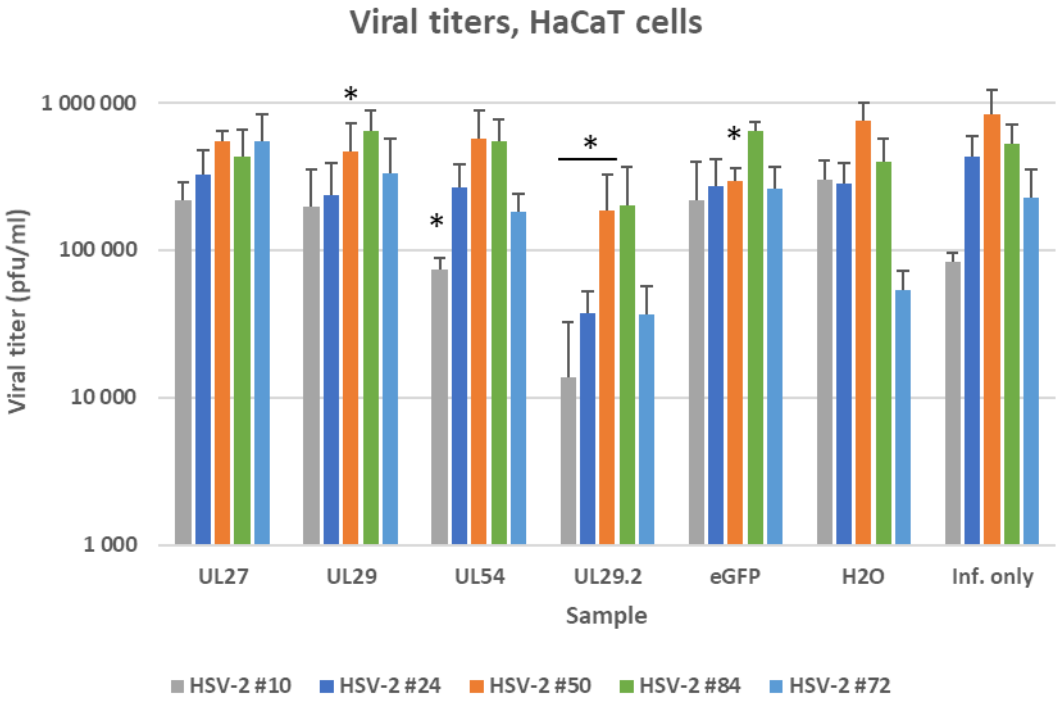

Figure 7.

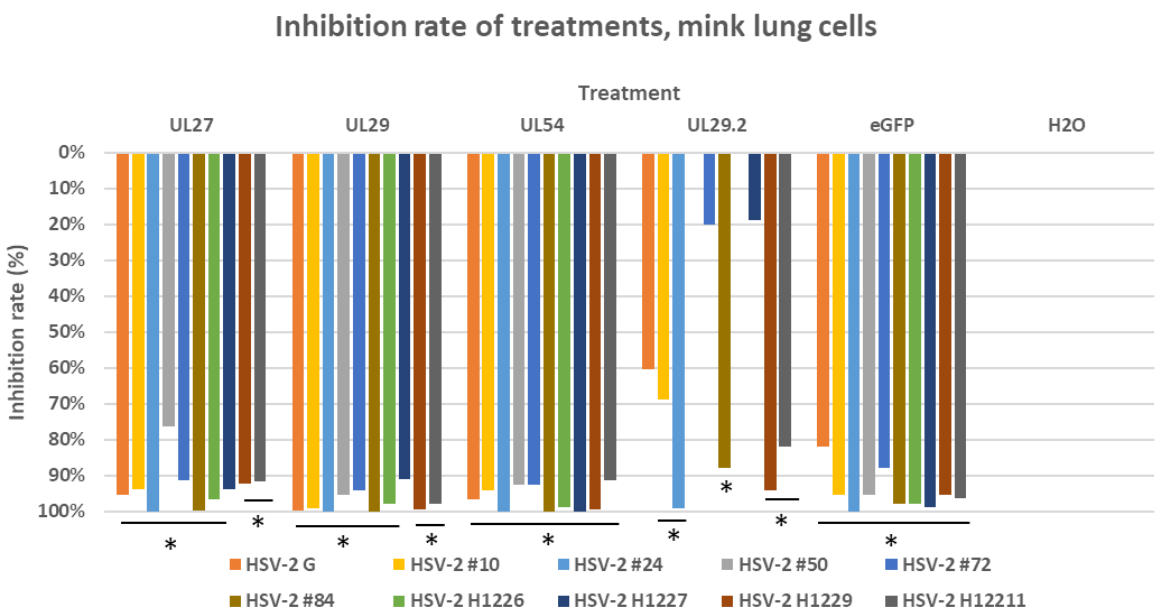

Figure 8.

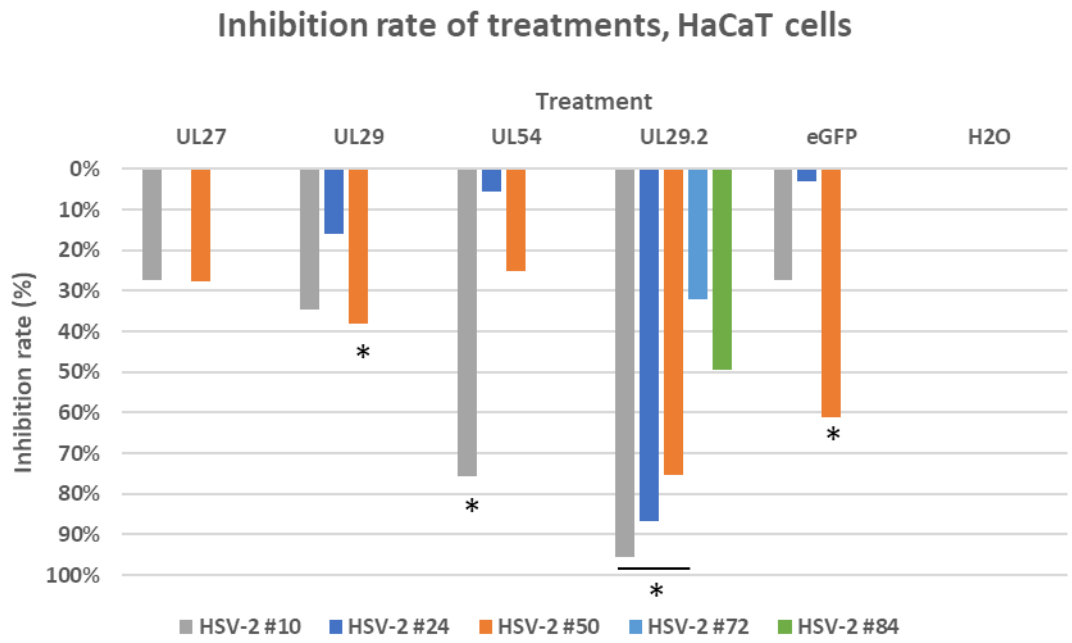

Figure 9.

Figures 6 – 9. The antiviral effects of enzymatically synthesized siRNA swarms in two virus infected cell lines. *The swarms target the essential HSV-2 genes UL27, UL29 and UL54 in MLC (Fig. 6 and 8) and HaCaT cells (Fig. 7 and 9) infected with one reference strain (G) or clinical isolates (#10, #24, #50, #72, #84, H1226, H1227, H1229, H12211) of HSV-2. Cells were also transfected with a non-specific eGFP swarm, a single site siRNA UL29.2 or water. The effects of siRNA swarms were determined by plaque titration in Vero cell culture from mink lung or HaCaT cell culture supernatants collected 48h post transfection. The results are presented as viral shedding in plaque*

*forming units (Fig. 6 – 7) and as relative inhibition rates (Fig. 8 – 9) of swarms and controls compared to infected, mock-treated cells (H2O), * = p<0,05.*

Because in some earlier experiments the UL27, UL29 and UL54 siRNA swarms had had a much higher antiviral efficacy in HaCaT cells, I suspected that the swarms were not being transfected into cells effectively. To test this hypothesis, the antiviral experiments were repeated with HaCaT cells using two HSV-2 strains, G and #72, and a lower cell confluency, 30% instead of the usual 60% used in previous studies. Cells were transfected with antiviral siRNA swarms (UL27, UL29 and UL54), the single site siRNA UL29.2 and controls (eGFP and water) and infected with the aforementioned HSV-2 strains 4 hours post transfection as was done in previous studies. In this experiment transfection appeared to be more successful as viral shedding decreased significantly in HaCaT cells treated with antiviral swarms compared to infected only and infected, mock-transfected cells (Fig. 10). The inhibition rate of swarms ranged between 79 and 93% (Fig. 11). As in previous experiments the single site UL29.2 siRNA had a higher inhibition rate than the antiviral swarms ranging from 92 to 94%. The non-specific eGFP swarm had the lowest inhibition rate ranging from 54 to 77%.

All in all, plaque titration results obtained from MLC and from the subsequent HaCaT experiments largely mirrored the predictions made based on the bioinformatics analyses. The UL27, UL29 and UL54 siRNA swarms were indeed all effective against the tested HSV-2 strains as is reflected in the high inhibition rates. However, results from the first HaCaT experiment were not in line with predictions of the swarms' efficacy, as inhibition rates were low and observed for only 3 of the 5 tested HSV-2 strains (#10, #24 and #50).

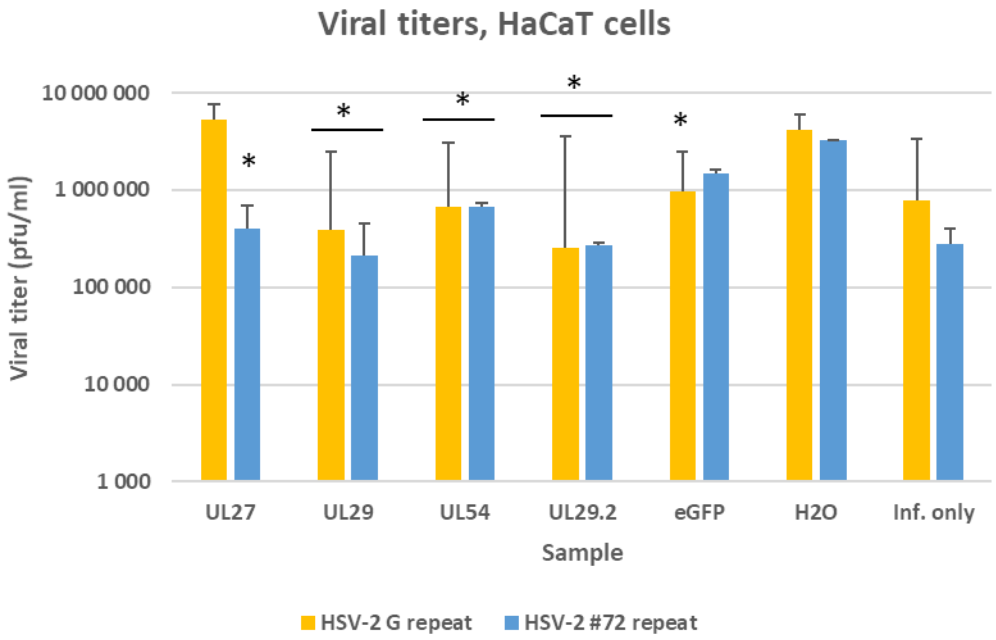

Figure 10.

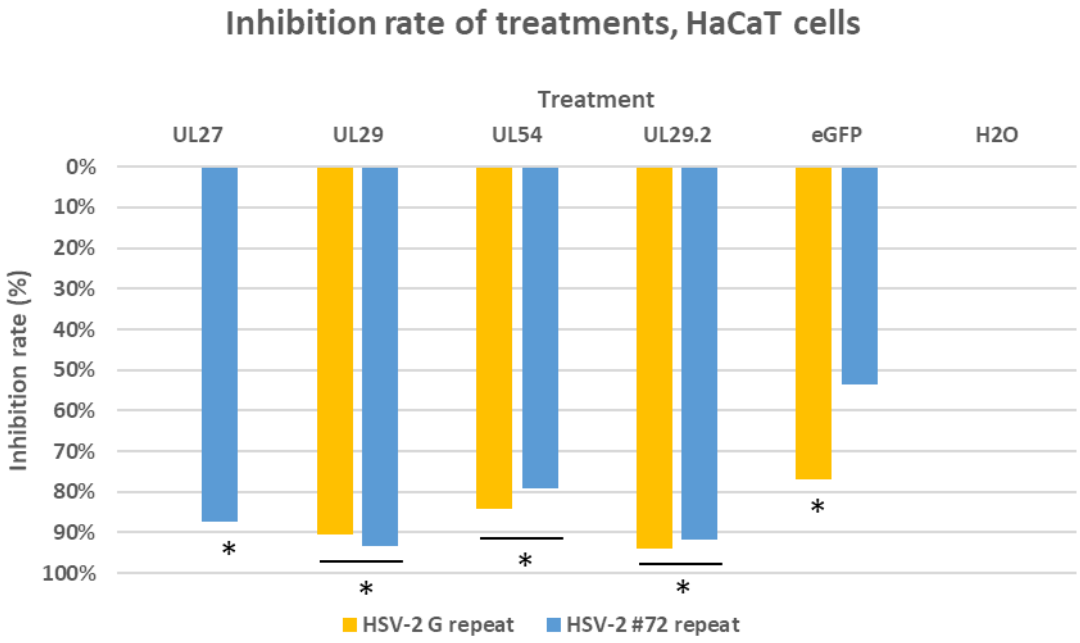

Figure 11.

Figures 10 – 11. The antiviral effects of enzymatically synthesized siRNA swarms in virus infected cells. *The swarms target the essential HSV-2 genes UL27, UL29 and UL54 in HaCaT cells infected with one reference strain (G) or one clinical isolate (#72) of HSV-2 when cell confluency is at 30%. Lower cell confluency was used to determine whether HaCaT cells would be more readily transfected and thus the effect of antiviral siRNA swarms would be higher. In addition to antiviral swarms, cells were also transfected with a non-specific eGFP swarm, a single site siRNA UL29.2 or water. The effects of siRNA swarms were determined by plaque titration in Vero cell culture from*

*HaCaT cell culture supernatants collected 48h post transfection. The results are presented as viral shedding in plaque forming units (Fig. 10) and as relative inhibition rates (Fig. 11) of swarms and controls compared to infected, mock-treated cells (H2O), * = p<0,05.*

4.3 qPCR results in MLC and HaCaT cells

To validate the results of antiviral studies conducted in HaCaT cells and MLC using several clinical HSV-2 strains and anti-HSV siRNA swarms, qPCR analysis was performed on cell supernatants collected 48 hours post transfection. Prior to this, cells had been first transfected with antiviral siRNA swarms targeting the essential viral genes *UL27*, *UL29* and *UL54* (swarms are also denoted UL27, UL29 and UL54), a non-specific eGFP swarm, a single site siRNA targeting the gene *UL29* called UL29.2 or water. 4 hours post transfection cells were infected with a reference strain (HSV-2 G) or a clinical HSV-2 isolate (H1226, H1227, H1229, H12211, #10, #24, #50, #72 or #84).

qPCR analysis yielded somewhat similar results to plaque titrations for select HSV-2 strains. In MLC several of the infected, UL27, UL29 and UL54 siRNA swarm treated cells appeared to have more HSV-2 *gD* gene copy numbers than infected only or infected, mock-treated cells, which means that swarm treated samples contained more virus (Fig. 12). The strains for which the antiviral swarm treatments seem to have been effective were HSV-2 G, #24, #50, #72 and #84, where inhibition rates ranged from 28 to 91% (Fig. 14). In cases where antiviral UL27, UL29 and UL54 swarms were effective in HaCaT cells, HSV-2 *gD* copy numbers were two to three-fold lower than infected, mock-treated cells (Fig. 13). Inhibition rates for antiviral swarms ranged from 3 to 73% for HSV-2 strains #10, #24, #50 and #72 (Fig. 15).

When comparing MLC titration results to qPCR analysis done for the same cell line, the single site siRNA UL29.2 had an inhibitory effect on more HSV-2 clinical strains than the antiviral siRNA swarms. The UL29.2 siRNA induced a two-fold decrease in HSV-2 *gD* gene copy numbers compared to infected, mock-treated cells (Fig. 12) and its inhibition rate for all HSV-2 strains ranged from 29 to 99% and more specifically 74 – 99% for six of the ten tested viral strains (Fig. 14). Somewhat similarly in HaCaT cells the single site siRNA had an antiviral effect on 4 of the 5 tested HSV-2 clinical isolates where inhibition rates ranged from 21 to 85 % (Fig. 15). For HSV-2 #10 and #50 the UL29.2 siRNA was more effective at inhibiting the virus than the UL27, UL29 and UL54 swarms.

As with the MLC titration results, qPCR analysis indicated the non-specific eGFP swarm had an antiviral effect on HSV-2 in MLC but only on 4 of the 10 tested HSV-2 strains as opposed to all

strains according to the titration results. The inhibition rates for these four HSV-2 strains ranged from 15% to 87% (Fig. 14). In HaCaT cells the eGFP swarm had an inhibitory effect on all tested HSV-2 strains and the inhibition rates ranged from 9 to 66% (Fig. 15).

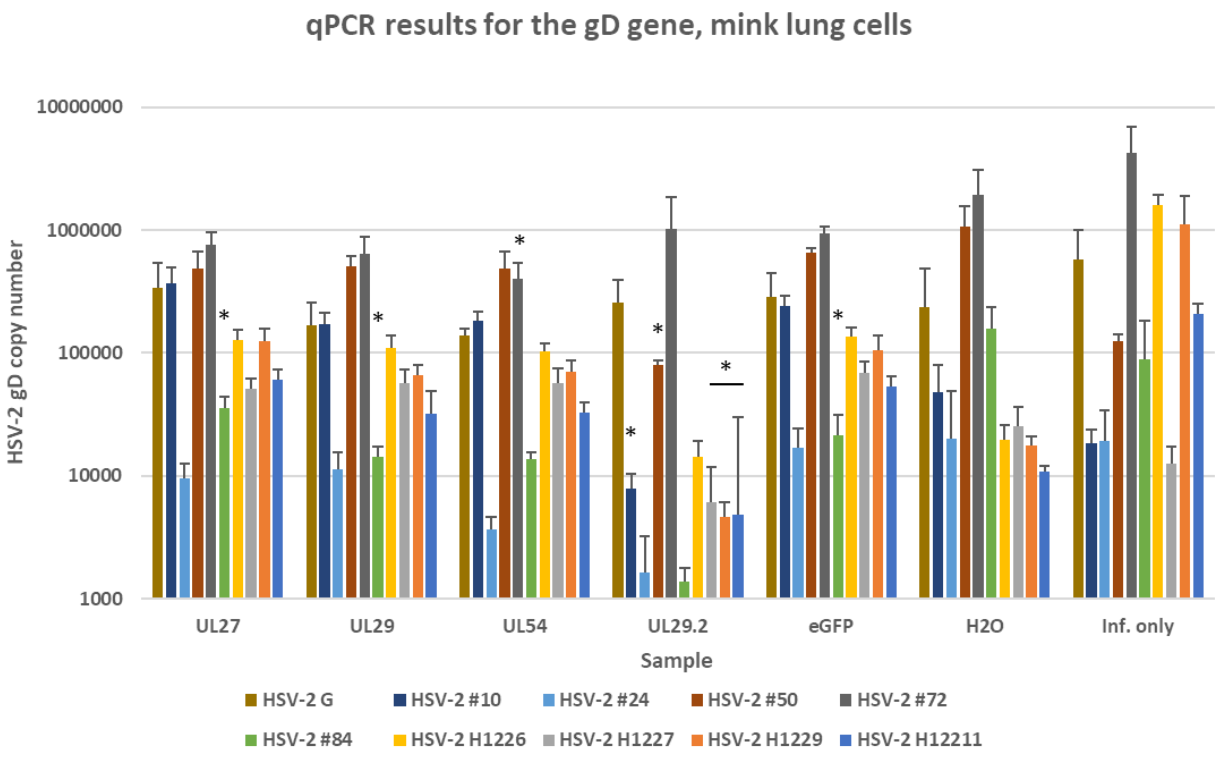

Figure 12.

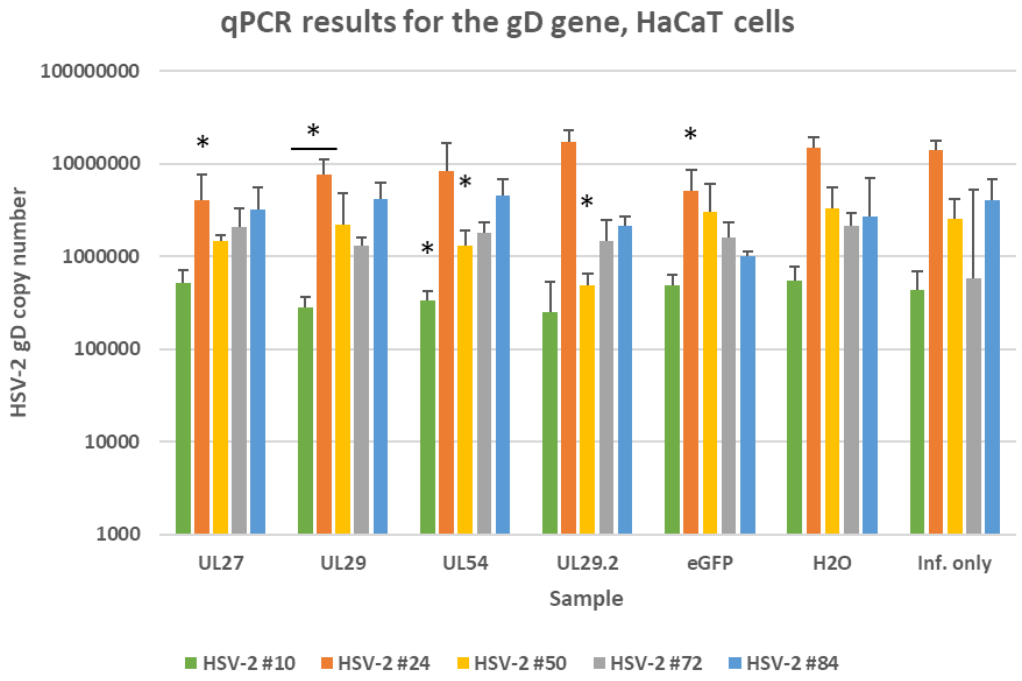

Figure 13.

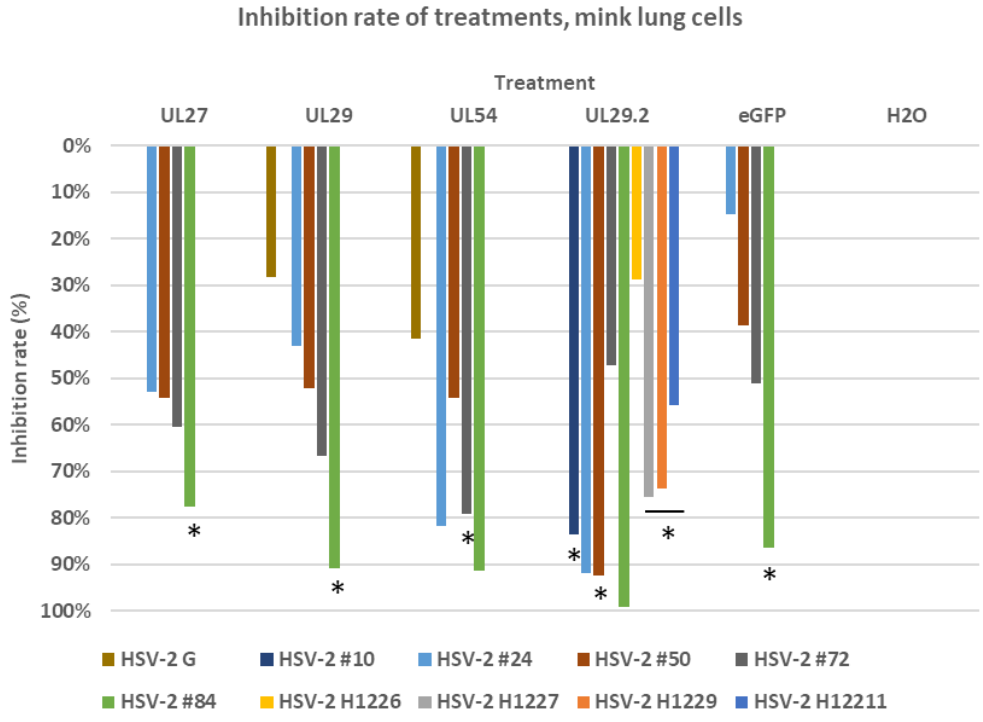

Figure 14.

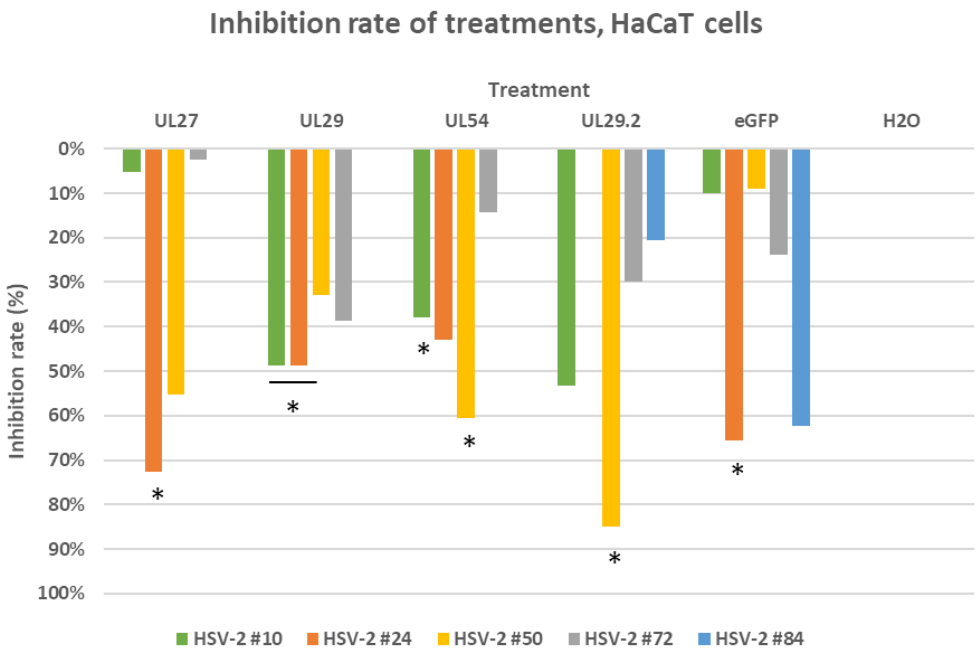

Figure 15.

Figure 12 – 15. The antiviral effects, determined by qPCR, of enzymatically synthesized siRNA swarms in two virus infected cell lines. *The swarms target the essential HSV-2 genes UL27, UL29 and UL54 in MLC (Fig. 12 and 14) and HaCaT (Fig. 13 and 15) cells infected with a reference strain (G) or one of several clinical isolates (#10, #24, #50, #72, #84, H1226, H1227, H1229, H12211) of HSV-2. Cells were also transfected with a non-specific eGFP swarm, a single site siRNA UL29.2 or water. The effects of the siRNA swarms were determined by qPCR for the HSV-2 gD gene DNA from mink lung or HaCaT cell culture supernatants collected 48h post transfection. The results are presented as HSV-2 gD DNA copy numbers in antiviral swarm (UL27, UL29 and UL54) or control (UL29.2, eGFP and H2O) treated and infected only cells (Fig. 12 – 13) and as relative inhibition rates of swarm and control treated samples compared to infected mock-treated (H2O) cells (Fig. 14 – 15), * = p<0,05.*

As mentioned before, the antiviral experiments were repeated in HaCaT cells with a lower cell confluency (30%) to see whether cells would be transfected more efficiently and whether antiviral siRNA swarms would have a higher inhibitory effect on HSV-2 strains as a result. According to qPCR analysis all anti-HSV siRNA swarms had a significant inhibitory effect on the HSV-2 strains as, the HSV-2 *gD* gene copy numbers decreased by nearly tenfold compared to infected, mock-treated cells for both HSV-2 G and #72 (Fig. 16). The inhibition rates of the UL27, UL29 and UL54 swarms ranged between 88 and 95% (Fig. 17). The single site siRNA UL29.2 was only slightly more effective

at inhibiting both HSV-2 strains with its inhibition rates being 92 and 98%. The non-specific eGFP swarm also had an inhibitory effect on the viral strains, with HSV-2 *gD* copy numbers decreasing 4 to 8-fold from infected mock-treated cells (Fig. 16). At 74 and 89% the inhibition rates for the eGFP swarm were lower than those of the anti-HSV swarms and the single site UL29.2 siRNA (Fig. 17). Collectively qPCR results from both MLC and HaCaT experiments were in line with predictions made using bioinformatics analyses to a varying degree. Much like the plaque titration results from the second HaCaT experiment, qPCR analysis yielded a similar outcome, where inhibition rates for the antiviral siRNA swarms UL27, UL29 and UL54 were high against both tested HSV-2 strains (G and #72.). The first HaCaT experiment yielded moderate inhibition rates for the antiviral swarms against 4 of the 5 of the tested HSV-2 strains (#10, #24, #50 and #72) but the rates were lower than expected based on predictions. Lastly for MLC the UL27, UL29 and UL54 swarms were effective against 5 of the 10 tested HSV-2 strains (G, #24, #50, #72 and #84) at best, while bioinformatics analyses predicted that swarms would be highly efficacious against all tested strains.

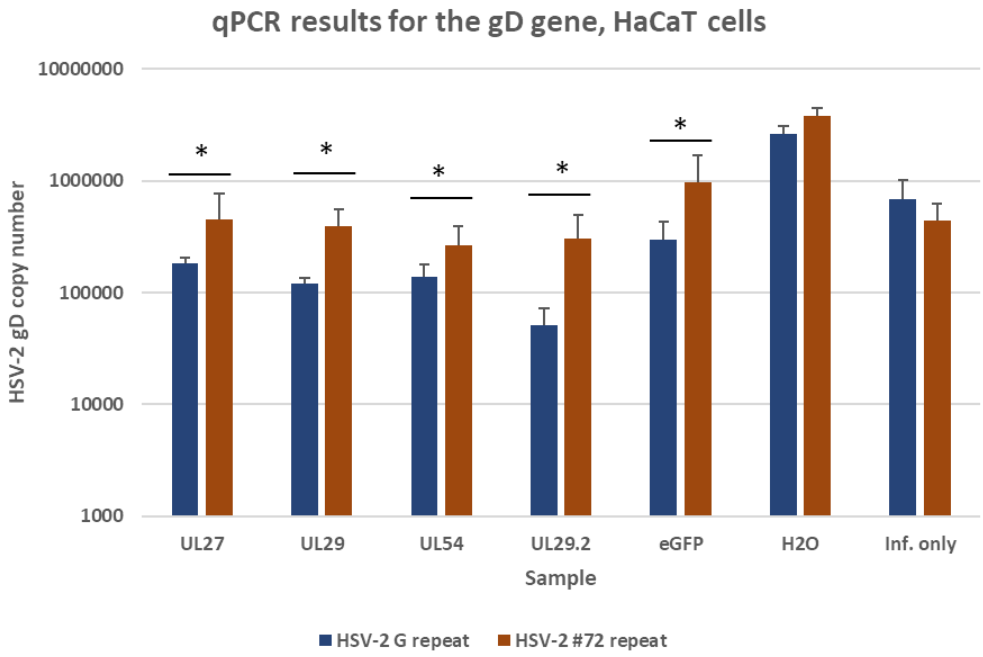

Figure 16.

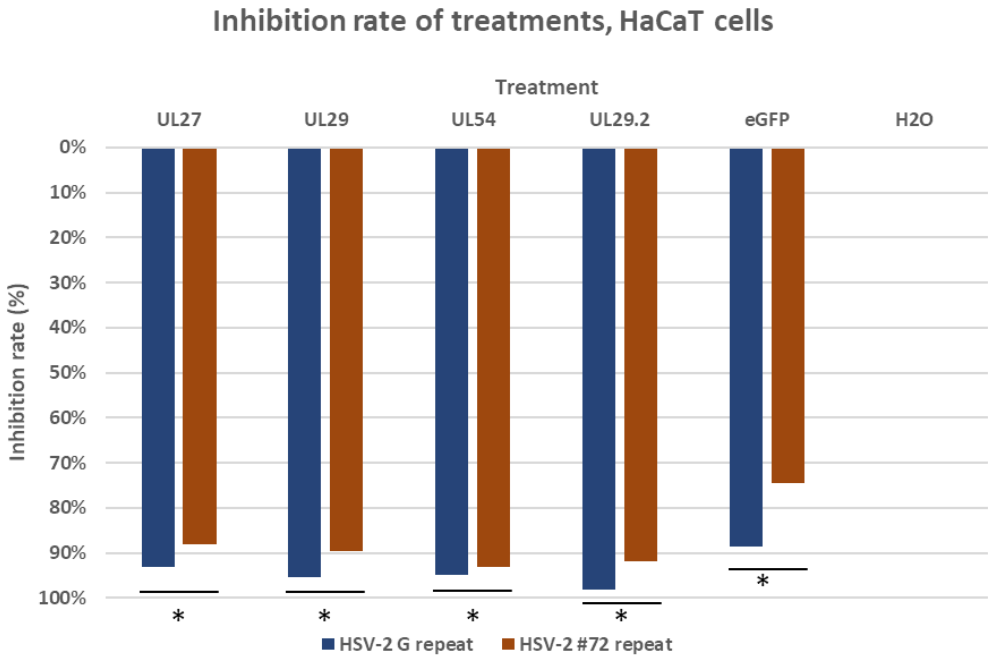

Figure 17.

Figure 16 – 17. The antiviral effects, determined by qPCR, of enzymatically synthesized siRNA swarms in virus infected cells. *The swarms target the essential HSV-2 genes UL27, UL29 and UL54 in HaCaT cells infected with a reference strain (G) or one clinical isolate (#72) of HSV-2 when cell confluency is at 30%. Lower cell confluency was used to determine whether HaCaT cells would be more readily transfected and thus the effect of antiviral siRNA swarms would be higher. Cells were also transfected with a non-specific eGFP swarm, a single site siRNA UL29.2 or water. The effects of siRNA swarms were determined by qPCR for the HSV-2 gD gene DNA from HaCaT cell culture supernatants collected 48h post transfection. The results are presented as HSV-2 gD DNA copy numbers in antiviral swarm (UL27, UL29 and UL54) or control (UL29.2, eGFP and H2O) treated and infected only cells (Fig. 16) and as relative inhibition rates of swarm and control treated samples compared to infected mock-treated (H2O) cells (Fig. 17), * = p<0,05.*

5 Discussion

HSV-1 and HSV-2 pose a significant burden on the human population with the number of people infected by these viruses exceeding several billion worldwide (WHO 2020). A novel treatment method which utilizes enzymatically synthesized siRNA swarms has previously been used to inhibit HSV-1 strains by targeting the essential viral genes *UL27*, *UL29* and *UL54 in vitro* and *in vivo* with promising results (Paavilainen 2017). Prior to that, a single site siRNA targeting the gene *UL29* had been developed to inhibit HSV-2 (Palliser et al. 2006). Because the use of siRNA pools offers several benefits over single site siRNAs and because HSV-2 contains the same genes that were targeted by siRNA swarms in earlier studies, it would be convenient from a therapeutic standpoint if the same siRNA swarms could inhibit clinical HSV-2 strains. This way one RNAi based drug could be developed to treat infections caused by either of the HSV viruses.

Bioinformatics tools were used to predict the antiviral effect of enzymatically synthesized siRNA swarms on HSV-2 by comparing the sequences of siRNA swarm target sites in HSV-1 strain 17+ to corresponding sites in several clinical and laboratory strains of HSV-2 using multiple sequence alignment tools. To test the actual antiviral effects of swarms against HSV-2 clinical strains, siRNA swarms were transfected into MLC and HaCaT cells with a dose of 10 pmol prior to infection and supernatants were later titered to establish the viral titer in plaque forming units. A *UL29* gene targeting single site siRNA, previously developed by Palliser et al. (2006) to combat HSV-2 infections, was also included in the study as a positive control.

Based on plaque titration results in HaCaT cells, in most cases *UL27*, *UL29* and *UL54* gene targeting siRNA swarms did not have a significant antiviral effect on HSV-2, which was in contrast with results obtained from MLC antiviral experiments. This raised the suspicion that siRNA swarms were not being effectively transfected into HaCaT cells, which prompted me to repeat the study using lower cell confluency. Thus 30% confluent HaCaT cells were transfected with the same anti-HSV siRNA swarms, single site siRNA, non-specific swarm or water and later infected with HSV-2 strain G and clinical isolate #72. In addition to plaque titration the effects of antiviral swarms on HSV-2 strains were confirmed through qPCR analysis, where supernatants collected 48 post transfection were amplified for the HSV-2 *gD* gene.

Bioinformatics analysis revealed that the UL29 siRNA swarm target site in HSV-1 strain 17+ was most identical to its counterparts in the tested HSV-2 strains with 95% sequence identity, followed

by target sites for the *UL27* and *UL54* gene targeting swarms that had 94% and ~78% sequence identity respectively (Table 2). Based on these results I predicted that the *UL27* and *UL29* gene targeting siRNA swarms would have the highest antiviral effect on HSV-2 strains with little difference in their efficacy when compared to each other. The *UL54* targeting swarm would have the least efficacious antiviral effect and there would be a visible difference in its ability to inhibit HSV-2 strains compared to the other two swarms.

Somewhat contrary to the aforementioned predictions, all anti-HSV siRNA swarms proved effective at inhibiting HSV-2 clinical isolates in MLC according to plaque titration results. This was proven by the decrease in viral titer (Fig. 6) and by inhibition rates reaching over 90% in almost all studied cases for all antiviral swarms (Fig. 8). Thus, the *UL54* targeting swarm was not considerably less effective at inhibiting HSV-2 compared to the other swarms. As predicted, compared to the other anti-HSV siRNA swarms the *UL29* targeting swarm reached higher inhibition rates more consistently for individual HSV-2 strains, but the difference in inhibition rates between the different anti-HSV swarms was minor.

Initially in HaCaT cells, contrary to what bioinformatics analyses suggested, swarms did not exhibit antiviral efficacy against all studied HSV-2 strains and when they did they reached lower inhibition rates (6 – 76%) than in MLC (Fig. 9). In the subsequent HaCaT antiviral study performed with lower cell confluency titration results showed that inhibition rates from antiviral swarms ranged from 79 to 93% (Fig. 11). These results were more in line with ones obtained from MLC antiviral studies and with predictions made using bioinformatics tools. Overall, based on plaque titration results no swarm could be declared a clear winner at inhibiting HSV-2 in either HaCaT cells or MLC, as no remarkable difference in their performance was observed.

Interestingly, according to titration results, in MLC the *UL29* gene targeting single site siRNA UL29.2 was visibly less effective at inhibiting HSV-2 than the siRNA swarms. The single site siRNA only reached significant levels of viral inhibition for 5 out of 10 HSV-2 strains (Fig. 8). Contrary to MLC however, in the initial HaCaT cell antiviral study the single site siRNA UL29.2 was more effective at inhibiting HSV-2 than any of the tested antiviral swarms (Fig. 9). The same result was observed in the subsequent HaCaT cell study where the single site siRNA was still more effective at inhibiting both HSV-2 #72 and G than the antiviral siRNA swarms but with a much less pronounced difference (Fig. 11).

According to qPCR analysis on the other hand, the single site siRNA UL29.2 had an antiviral effect on all HSV-2 strains except for strain G in MLC (Fig. 14). In the initial HaCaT antiviral experiment qPCR analysis showed that antiviral swarms were effective against all tested HSV-2 strains except for #84, whereas the single site siRNA UL29.2 had an antiviral effect on #84 and all other tested HSV-2 strains except strain #24 (Fig. 15). In the subsequent HaCaT cell study performed with lower cell confluency qPCR analysis showed that the *UL27*, *UL29* and *UL54* gene targeting swarms and the single site siRNA UL29.2 were all effective against the two tested HSV-2 strains (G and #72) (Fig. 17). In contrast to the MLC and HaCaT titration results, according to qPCR analysis the UL29 siRNA swarm did not perform better than any other swarm nor was there any consistent patterns visible in the results in the case of HaCaT cells. In MLC, when swarms exhibited antiviral effects the *UL54* gene targeting swarm reached slightly higher inhibition rates compared to the two other siRNA swarms (Fig. 14).

There was a seeming discrepancy between plaque titration and qPCR results obtained from both MLC and HaCaT antiviral studies, discussed already above. One important factor that may have affected the qPCR results is the one nucleotide mismatch in the HSV-2 *gD* reverse primer (HS2D-4U). The mismatch is in the 10th position from the 5' end of the primer where guanine in the primer is cytosine in the HSV-2 strains. Even single mismatches between primers and their target sequences are known to produce errors in qPCR results though this is less likely to occur when mismatches appear in the 5' end of the primer (Whiley and Sloots 2005).

As an example of discrepancies, in MLC qPCR results showed that antiviral siRNA swarms UL27, UL29 and UL54 had an antiviral effect on about half of the tested HSV-2 strains (Fig. 14), while titration results indicated that all swarms had a statistically significant and high antiviral effect on all tested HSV-2 strains with only a few exceptions (Fig. 8). In the initial HaCaT cell antiviral experiment the reverse was observed where qPCR results indicated that antiviral siRNA swarms had an inhibitory effect on 4 out of 5 tested HSV-2 strains (Fig. 15) while plaque titration showed that antiviral efficacy was observed in 3 out of the 5 tested HSV-2 strains (Fig. 9). Additionally, in the initial HaCaT experiments the inhibitory effect of swarms was generally lower than that seen in the qPCR analysis and overall, the siRNA swarm with the highest inhibitory effect on HSV-2 differed between titration and qPCR results. Interestingly the least amount of discrepancy between plaque titration and qPCR analysis was seen in the subsequent HaCaT antiviral study performed with lower cell confluency (Fig. 11 and 17). It must be noted however, that the qPCR for HSV DNA does not measure viral infectivity but only the presence of the U_S segment of the viral

DNA where the qPCR target, the HSV-2 *gD* gene, resides. Thus, the release of infectious virus is reflected in the titration results.

It was unexpected that the non-specific siRNA swarm eGFP, which does not target any sequence in HSV, had a relatively high inhibitory effect on HSV-2 in both HaCaT and MLC according to titration results. In MLC the eGFP swarm had a statistically significant antiviral effect on all HSV strains and inhibition rates ranged between 82 and 100% (Fig. 8). In HaCaT cells the antiviral effect of the eGFP swarm was present in 3 of the 5 of HSV-2 strains in the initial study (Fig. 9) and in both HSV-2 strains in the subsequent study (Fig. 11). However, the antiviral effect was statistically significant only for HSV-2 #50 and for HSV-2 G in the first and the second HaCaT antiviral experiment respectively. Results from qPCR analysis showed the same discrepancy as mentioned before when compared to plaque titration results. According to qPCR analysis in MLC eGFP had an inhibitory effect on 4 of the 10 tested HSV-2 strains (Fig. 14) whereas in the initial and subsequent HaCaT antiviral studies the eGFP swarm was effective against all tested HSV-2 strains (Fig. 15 and 17). To ascertain whether or not the eGFP swarm was faulty itself, another non-specific siRNA swarm called pET was also tested briefly alongside eGFP in MLC. The results remained the same, with the pET swarm yielding as high of an antiviral effect against the tested HSV-2 strains as eGFP.

The seemingly high antiviral effect of the non-specific eGFP swarm could be the result of the swarm inducing innate immunity especially in MLC, which then leads to a relatively strong inhibition of HSV replication. The innate immunity induced by the eGFP siRNA swarm has previously been studied in HaCaT cells, where it did not induce significant responses as such, in comparison to mock treated or non-transfected samples. When studied together with HSV-1 infection the eGFP swarm induced IFN expression similarly to the control treatments, which meant that the response was induced mainly by the virus. (Paavilainen et al. 2015.) Unfortunately, the innate immunity, especially IFN responses, could not be determined in MLC in the context of my study, because no qPCR test was available for markers of the mink IFN system. Thus, it remains plausible that the eGFP siRNA control swarm elicited high cellular responses in MLC, which limited viral replication and mimicked the sequence specific RNAi effect. As HSV is susceptible to IFNs in epithelial cells this effect could prevent us from seeing the true outcome of RNAi.

Taken together the results indicate that inhibition of different HSV strains is dependent on the cell line and viral strain under study, more than on the viral gene sequence selected as the target of siRNA swarms. This was proven by the fact that all anti-HSV siRNA swarms had a relatively similar inhibitory effect on HSV-2 despite their target sequences in the virus strains varying in identity.

While there is an unfortunate discrepancy between results obtained from plaque titration and qPCR analysis, titration results are more reliable and credible because they represent the genuine infectious virus instead of DNA fragments. The titers of infectious virus may be lower than they appear in the titration results of this study, because the immunoglobulin, used to prevent the appearance of secondary plaques during virus culturing, does not contain as many antibodies against HSV-2 as against HSV-1.

In future studies we should further elucidate the mechanism by which these antiviral siRNA swarms work. Studies should be conducted to determine whether the swarms induce innate immunity and whether their antiviral effect is caused by target gene inhibition through RNA interference, induced innate immunity preventing viral replication or by a combination of the two.

References

Aalto, A. P., Sarin, L. P., van Dijk, A. A., Saarma, M., Poranen, M. M., Arumae, U. & Bamford, D. H. (2007) Large-scale production of dsRNA and siRNA pools for RNA interference utilizing bacteriophage 6 RNA-dependent RNA polymerase. *RNA* **13**:422–429.

Adams, D., Gonzalez-Duarte, A., O'Riordan, W. D., Yang, C.-C., Ueda, M., Kristen, A. V., Tournev, I., Schmidt, H. H., Coelho, T., Berk, J. L., Lin, K.-P., Vita, G., Attarian, S., Planté-Bordeneuve, V., Mezei, M. M., Campistol, J. M., Buades, J., Brannagan, T. H., Kim, B. J. et al. (2018) Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med* **379**:11–21.

Adams, D., Suhr, O. B., Dyck, P. J., Litchy, W. J., Leahy, R. G., Chen, J., Gollob, J. & Coelho, T. (2017) Trial design and rationale for APOLLO, a Phase 3, placebo-controlled study of patisiran in patients with hereditary ATTR amyloidosis with polyneuropathy. *BMC Neurol* **17**:181.

Akinc, A., Querbes, W., De, S., Qin, J., Frank-Kamenetsky, M., Jayaprakash, K. N., Jayaraman, M., Rajeev, K. G., Cantley, W. L., Dorkin, J. R., Butler, J. S., Qin, L., Racie, T., Sprague, A., Fava, E., Zeigerer, A., Hope, M. J., Zerial, M., Sah, D. W. et al. (2010) Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol Ther* **18**:1357–1364.

Alvarez, R., Elbashir, S., Borland, T., Toudjarska, I., Hadwiger, P., John, M., Roehl, I., Morskaya, S. S., Martinello, R., Kahn, J., Van Ranst, M., Tripp, R. A., DeVincenzo, J. P., Pandey, R., Maier, M., Nechev, L., Manoharan, M., Kotelianski, V. & Meyers, R. (2009) RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy. *Antimicrob Agents Chemother* **53**:3952–3962.

Ando, Y., Coelho, T., Berk, J. L., Cruz, M. W., Ericzon, B.-G., Ikeda, S., Lewis, W. D., Obici, L., Planté-Bordeneuve, V., Rapezzi, C., Said, G. & Salvi, F. (2013) Guideline of transthyretin-related hereditary amyloidosis for clinicians. *Orphanet J Rare Dis* **8**:31.

Balfour, H. H. (1999) Antiviral Drugs. *N Engl J Med* **340**:1255–1268.

Bellner, L., Thorén, F., Nygren, E., Liljeqvist, J.-Å., Karlsson, A. & Eriksson, K. (2005) A proinflammatory peptide from herpes simplex virus type 2 glycoprotein G affects neutrophil, monocyte, and NK cell functions. *J Immunol* **174**:2235–2241.

Blake, S. J., Bokhari, F. F. & McMillan, N. A. J. (2012) RNA interference for viral infections. *Curr Drug Targets* **13**:1411–1420.

Bramsen, J. B., Laursen, M. B., Nielsen, A. F., Hansen, T. B., Bus, C., Langkjoør, N., Babu, B. R., Højland, T., Abramov, M., Van Aerschot, A., Odadzic, D., Smicius, R., Haas, J., Andree, C., Barman, J., Wenska, M., Srivastava, P., Zhou, C., Honcharenko, D. et al. (2009) A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res* **37**:2867–2881.

Brown, Z. A., Selke, S., Zeh, J., Kopelman, J., Maslow, A., Ashley, R. L., Watts, D. H., Berry, S., Herd, M. & Corey, L. (1997) The acquisition of herpes simplex virus during pregnancy. *N Engl J Med* **337**:509–516.

Brummelkamp, T. R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science (80-)* **296**:550–553.

Butler, J. S., Chan, A., Costelha, S., Fishman, S., Willoughby, J. L. S., Borland, T. D., Milstein, S.,

Foster, D. J., Gonçalves, P., Chen, Q., Qin, J., Bettencourt, B. R., Sah, D. W., Alvarez, R., Rajeev, K. G., Manoharan, M., Fitzgerald, K., Meyers, R. E., Nochur, S. V. et al. (2016) Preclinical evaluation of RNAi as a treatment for transthyretin-mediated amyloidosis. *Amyloid* **23**:109–118.

Campadelli-Fiume, G., Cocchi, F., Menotti, L. & Lopez, M. (2000) The novel receptors that mediate the entry of herpes simplex viruses and animal alphaherpesviruses into cells. *Rev Med Virol* **10**:305–319.

Centers for Disease Control and Prevention (2010) Herpes simplex. Available at: https://en.wikipedia.org/wiki/Herpes_simplex (Accessed: 9 April 2021).

Chen, X., Qian, Y., Yan, F., Tu, J., Yang, X., Xing, Y. & Chen, Z. (2013) 5'-Triphosphate-siRNA activates RIG-I-dependent type i interferon production and enhances inhibition of hepatitis B virus replication in HepG2.2.15 cells. *Eur J Pharmacol* **721**:86–95.

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. & Shiekhattar, R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**:740–744.

Chin, W. X., Ang, S. K. & Chu, J. J. H. (2017) Recent advances in therapeutic recruitment of mammalian RNAi and bacterial CRISPR-Cas DNA interference pathways as emerging antiviral strategies. *Drug Discov Today* **22**:17–30.

Christophers, J., Clayton, J., Craske, J., Ward, R., Collins, P., Trowbridge, M. & Darby, G. (1998) Survey of resistance of herpes simplex virus to acyclovir in northwest England. *Antimicrob Agents Chemother* **42**:868–872.

Coelho, T., Adams, D., Silva, A., Lozeron, P., Hawkins, P. N., Mant, T., Perez, J., Chiesa, J., Warrington, S., Tranter, E., Munisamy, M., Falzone, R., Harrop, J., Cehelsky, J., Bettencourt, B. R., Geissler, M., Butler, J. S., Sehgal, A., Meyers, R. E. et al. (2013) Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *N Engl J Med* **369**:819–829.

Cohen, J. C., Boerwinkle, E., Mosley, T. H. & Hobbs, H. H. (2006) Sequence variations in PCSK9*,* low LDL, and protection against coronary heart disease. *N Engl J Med* **354**:1264–1272.

Darougar, S., Wishart, M. S. & Viswalingam, N. D. (1985) Epidemiological and clinical features of primary herpes simplex virus ocular infection. *Br J Ophthalmol* **69**:2–6.

DeVincenzo, J., Lambkin-Williams, R., Wilkinson, T., Cehelsky, J., Nochur, S., Walsh, E., Meyers, R., Gollob, J. & Vaishnaw, A. (2010) A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. *Proc Natl Acad Sci* **107**:8800–8805.

DiGiusto, D. L., Krishnan, A., Li, L., Li, H., Li, S., Rao, A., Mi, S., Yam, P., Stinson, S., Kalos, M., Alvarnas, J., Lacey, S. F., Yee, J.-K., Li, M., Couture, L., Hsu, D., Forman, S. J., Rossi, J. J. & Zaia, J. A. (2010) RNA-based gene therapy for HIV with lentiviral vector-modified CD34+ cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci Transl Med* **2**:36ra43-36ra43.

Dunning, J., Sahr, F., Rojek, A., Gannon, F., Carson, G., Idriss, B., Massaquoi, T., Gandi, R., Joseph, S., Osman, H. K., Brooks, T. J. G., Simpson, A. J. H., Goodfellow, I., Thorne, L., Arias, A., Merson, L., Castle, L., Howell-Jones, R., Pardinaz-Solis, R. et al. (2016) Experimental treatment of Ebola virus disease with TKM-130803: a single-arm Phase 2 clinical trial. *PLoS Med* **13**:e1001997.

Ebert, G., Poeck, H., Lucifora, J., Baschuk, N., Esser, K., Esposito, I., Hartmann, G. & Protzer, U. (2011) 5' Triphosphorylated small interfering RNAs control replication of hepatitis B virus and induce an interferon response in human liver cells and mice. *Gastroenterology* **141**:696-706.e3.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**:494–498.

Eulalio, A., Huntzinger, E. & Izaurralde, E. (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol* **15**:346–353.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in caenorhabditis elegans. *Nature* **391**:806–811.

Fries, L. F., Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Hammer, C. H. & Frank, M. M. (1986) Glycoprotein C of herpes simplex virus 1 is an inhibitor of the complement cascade. *J Immunol* **137**:1636–41.

Früh, K., Ahn, K., Djaballah, H., Sempé, P., van Endert, P. M., Tampé, R., Peterson, P. A. & Yang, Y. (1995) A viral inhibitor of peptide transporters for antigen presentation. *Nature* **375**:415–418.

Gantier, M. P. (2014) Processing of double-stranded RNA in mammalian cells: A direct antiviral role?. *J Interf Cytokine Res* **34**:469–477.

Ge, Q., Dallas, A., Ilves, H., Shorenstein, J., Behlke, M. A. & Johnston, B. H. (2010) Effects of chemical modification on the potency, serum stability, and immunostimulatory properties of short shRNAs. *RNA* **16**:118–130.

Gibbings, D. J., Ciaudo, C., Erhardt, M. & Voinnet, O. (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* **11**:1143–1149.

Gitlin, L., Stone, J. K. & Andino, R. (2005) Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. *J Virol* **79**:1027–1035.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**:235–240.

Grünewald, K., Desai, P., Winkler, D. C., Heymann, J. B., Belnap, D. M., Baumeister, W. & Steven, A. C. (2003) Three-dimensional structure of herpes simplex virus from cryo-electron tomography. *Science (80-)* **302**:1396–1398.

Haase, A. D., Jaskiewicz, L., Zhang, H., Lainé, S., Sack, R., Gatignol, A. & Filipowicz, W. (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep* **6**:961–967.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. (1975) Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. *Proc Natl Acad Sci U S A* **72**:4243–4247.

He, B., Gross, M. & Roizman, B. (1997) The 134.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1 to dephosphorylate the subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci* **94**:843–848.

Hill, A., Jugovic, P., York, L., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. & Johnson, D. (1995) Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**:411–415.

Holmgren, G., Steen, L., Ekstedt, J., Groth, C.-G., Ericzon, B.-G., Eriksson, S., Andersen, O., Karlberg,

I., Nordén, G., Nakazato, M., Hawkins, P., Richardson, S. & Pepys, M. (2008) Biochemical effect of liver transplantation in two Swedish patients with familial amyloidotic polyneuropathy (FAP-met30). *Clin Genet* **40**:242–246.

Hook, L. M., Huang, J., Jiang, M., Hodinka, R. & Friedman, H. M. (2008) Blocking antibody access to neutralizing domains on glycoproteins involved in entry as a novel mechanism of immune evasion by herpes simplex virus type 1 glycoproteins C and E. *J Virol* **82**:6935–6941.

Huang, Y., Hong, J., Zheng, S., Ding, Y., Guo, S., Zhang, H., Zhang, X., Du, Q. & Liang, Z. (2011) Elimination pathways of systemically delivered siRNA. *Mol Ther* **19**:381–385.

Hukkanen, V. (2020) Herpesvirusten yleisiä ominaisuuksia - Duodecim Oppiportti. Available at: https://www.oppiportti.fi/op/mbg00405/do (Accessed: 10 February 2021).

Hukkanen, V. & Seppänen, M. (2020) Herpes simplex -virukset - Duodecim Oppiportti. Available at: https://www.oppiportti.fi/op/mbg00406/do#s2 (Accessed: 4 February 2021).

James, C., Harfouche, M., Welton, N. J., Turner, K. M. E., Abu-Raddad, L. J., Gottlieb, S. L. & Looker, K. J. (2020) Herpes simplex virus: Global infection prevalence and incidence estimates, 2016. *Bull World Health Organ* **98**:315–329.

Janas, M. M., Schlegel, M. K., Harbison, C. E., Yilmaz, V. O., Jiang, Y., Parmar, R., Zlatev, I., Castoreno, A., Xu, H., Shulga-Morskaya, S., Rajeev, K. G., Manoharan, M., Keirstead, N. D., Maier, M. A. & Jadhav, V. (2018) Selection of GalNAc-conjugated siRNAs with limited off-target-driven rat hepatotoxicity. *Nat Commun* **9**:1–10.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M. & Stow, N. D. (1988) Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *J Virol* **62**:1347–54.

Johnston, C., Gottlieb, S. L. & Wald, A. (2016) Status of vaccine research and development of vaccines for herpes simplex virus. *Vaccine* **34**:2948–2952.

Jurak, I., Kramer, M. F., Mellor, J. C., van Lint, A. L., Roth, F. P., Knipe, D. M. & Coen, D. M. (2010) Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. *J Virol* **84**:4659–4672.

Kanasty, R., Dorkin, J. R., Vegas, A. & Anderson, D. (2013) Delivery materials for siRNA therapeutics. *Nat Mater* **12**:967–977.

Khvorova, A. & Watts, J. K. (2017) The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotechnol* **35**:238–248.

Kieff, E., Hoyer, B., Bachenheimer, S. & Roizman, B. (1972) Genetic relatedness of type 1 and type 2 herpes simplex viruses. *J Virol* **9**:738–45.

Kim, D. H., Behlke, M. A., Rose, S. D., Chang, M. S., Choi, S. & Rossi, J. J. (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* **23**:222–226.

Kim, D. H., Longo, M., Han, Y., Lundberg, P., Cantin, E. & Rossi, J. J. (2004) Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat Biotechnol* **22**:321–325.

Labetoulle, M., Auquier, P., Conrad, H., Crochard, A., Daniloski, M., Bouée, S., El Hasnaoui, A. & Colin, J. (2005) Incidence of herpes simplex virus keratitis in France. *Ophthalmology* **112**:888–895.

Langston, A. A., Redei, I., Caliendo, A. M., Somani, J., Hutcherson, D., Lonial, S., Bucur, S., Cherry, J.,

Allen, A. & Waller, E. K. (2002) Development of drug-resistant herpes simplex virus infection after haploidentical hematopoietic progenitor cell transplantation. *Blood* **99**:1085–1088.

Layzer, J. M., McCaffrey, A. P., Tanner, A. K., Huang, Z., Kay, M. A. & Sullenger, B. A. (2004) In vivo activity of nuclease-resistant siRNAs. *RNA* **10**:766–771.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. & Kim, V. N. (2002) MicroRNA maturation: Stepwise processing and subcellular localization. *EMBO J* **21**:4663–4670.

Levanova, A. A., Kalke, K. M., Lund, L. M., Sipari, N., Sadeghi, M., Nyman, M. C., Paavilainen, H., Hukkanen, V. & Poranen, M. M. (2020) Enzymatically synthesized 2′-fluoro-modified Dicer-substrate siRNA swarms against herpes simplex virus demonstrate enhanced antiviral efficacy and low cytotoxicity. *Antiviral Res* **182**:104916.

Levanova, A. & Poranen, M. M. (2018) RNA interference as a prospective tool for the control of human viral infections. *Front Microbiol* **9**:2151.

Lewis, B. P., Burge, C. B. & Bartel, D. P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**:15–20.

Li, F. & Ding, S.-W. (2006) Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annu Rev Microbiol* **60**:503–531.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. & Hannon, G. J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science (80-)* **305**:1437–1441.

Liu, J., Rivas, F. V., Wohlschlegel, J., Yates, J. R., Parker, R. & Hannon, G. J. (2005) A role for the P-body component GW182 in microRNA function. *Nat Cell Biol* **7**:1161–1166.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J. & Parker, R. (2005) MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**:719–723.

Looker, K. J., Elmes, J. A. R., Gottlieb, S. L., Schiffer, J. T., Vickerman, P., Turner, K. M. E. & Boily, M. C. (2017) Effect of HSV-2 infection on subsequent HIV acquisition: An updated systematic review and meta-analysis. *Lancet Infect Dis* **17**:1303–1316.

Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M. E., Vickerman, P., Gottlieb, S. L. & Newman, L. M. (2015) Global and regional estimates of prevalent and incident herpes simplex virus type 1 infections in 2012. *PLoS One* **10**:e0140765.

Makeyev, E. V. (2000) Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage phi 6. *EMBO J* **19**:124–133.

Malek, A., Merkel, O., Fink, L., Czubayko, F., Kissel, T. & Aigner, A. (2009) In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(-PEG)/siRNA complexes. *Toxicol Appl Pharmacol* **236**:97–108.

Maniataki, E. & Mourelatos, Z. (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes Dev* **19**:2979–2990.

Marcocci, M. E., Napoletani, G., Protto, V., Kolesova, O., Piacentini, R., Li Puma, D. D., Lomonte, P., Grassi, C., Palamara, A. T. & De Chiara, G. (2020) Herpes simplex virus-1 in the brain: The dark side of a sneaky infection. *Trends Microbiol* **28**:808–820.

Margolis, D. M., Rabson, A. B., Straus, S. E. & Ostrove, J. M. (1992) Transactivation of the HIV-1 LTR

by HSV-1 immediate-early genes. *Virology* **186**:788–791.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P. & Zamore, P. D. (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**:607–620.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. & Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* **15**:185–197.

Mettenleiter, T. C., Müller, F., Granzow, H. & Klupp, B. G. (2013) The way out: What we know and do not know about herpesvirus nuclear egress. *Cell Microbiol* **15**:170–178.

Morfin, F., Bilger, K., Boucher, A., Thiebaut, A., Najioullah, F., Bleyzac, N., Raus, N., Bosshard, S., Aymard, M., Michallet, M. & Thouvenot, D. (2004) HSV excretion after bone marrow transplantation: A 4-year survey. *J Clin Virol* **30**:341–345.

Paavilainen, H. (2017) Inhibition of herpes simplex infection with RNA interference. University of Turku. Faculty of Medicine, Department of Virology. Doctoral dissertation.

Paavilainen, H., Lehtinen, J., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. (2016) Inhibition of clinical pathogenic herpes simplex virus 1 strains with enzymatically created siRNA pools. *J Med Virol* **88**:2196–2205.

Paavilainen, H., Lehtinen, J., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. (2017) Topical treatment of herpes simplex virus infection with enzymatically created siRNA swarm. *Antivir Ther* **22**:631–637.

Paavilainen, H., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. (2015) Innate responses to small interfering RNA pools inhibiting herpes simplex virus infection in astrocytoid and epithelial cells. *Innate Immun* **21**:349–357.

Palliser, D., Chowdhury, D., Wang, Q.-Y., Lee, S. J., Bronson, R. T., Knipe, D. M. & Lieberman, J. (2006) An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* **439**:89–94.

Parmar, R., Willoughby, J. L. S., Liu, J., Foster, D. J., Brigham, B., Theile, C. S., Charisse, K., Akinc, A., Guidry, E., Pei, Y., Strapps, W., Cancilla, M., Stanton, M. G., Rajeev, K. G., Sepp-Lorenzino, L., Manoharan, M., Meyers, R., Maier, M. A. & Jadhav, V. (2016) 5′-(*E*)-Vinylphosphonate: A stable phosphate mimic can improve the RNAi activity of siRNA-GalNAc conjugates. *ChemBioChem* **17**:985–989.

Perng, G. C., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A., Slanina, S. M., Hofman, F. M., Ghiasi, H., Nesburn, A. B. & Wechsler, S. L. (2000) Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. *Science* **287**:1500–1503.

Piret, J. & Boivin, G. (2011) Resistance of herpes simplex viruses to nucleoside analogues: Mechanisms, prevalence, and management. *Antimicrob Agents Chemother* **55**:459–472.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. & Khvorova, A. (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* **22**:326–330.

Robbins, M., Judge, A., Liang, L., McClintock, K., Yaworski, E. & MacLachlan, I. (2007) 2′-O-methyl-modified RNAs act as TLR7 antagonists. *Mol Ther* **15**:1663–1669.

Roizman, B. & Whitley, R. J. (2013) An inquiry into the molecular basis of HSV latency and reactivation. *Annu Rev Microbiol* **67**:355–374.

Romanovskaya, A., Paavilainen, H., Nygårdas, M., Bamford, D. H., Hukkanen, V. & Poranen, M. M. (2012) Enzymatically produced pools of canonical and Dicer-substrate siRNA molecules display comparable gene silencing and antiviral activities against herpes simplex virus. *PLoS One* **7**:e51019.

Romanovskaya, A., Sarin, L. P., Bamford, D. H. & Poranen, M. M. (2013) High-throughput purification of double-stranded RNA molecules using convective interaction media monolithic anion exchange columns. *J Chromatogr A* **1278**:54–60.

Rowczenio, D. M., Noor, I., Gillmore, J. D., Lachmann, H. J., Whelan, C., Hawkins, P. N., Obici, L., Westermark, P., Grateau, G. & Wechalekar, A. D. (2014) Online registry for mutations in hereditary amyloidosis including nomenclature recommendations. *Hum Mutat* **35**:E2403–E2412.

Rowe, A. M., St. Leger, A. J., Jeon, S., Dhaliwal, D. K., Knickelbein, J. E. & Hendricks, R. L. (2013) Herpes keratitis. *Prog Retin Eye Res* **32**:88–101.

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**:199–208.

Seo, G. J., Kincaid, R. P., Phanaksri, T., Burke, J. M., Pare, J. M., Cox, J. E., Hsiang, T. Y., Krug, R. M. & Sullivan, C. S. (2013) Reciprocal inhibition between intracellular antiviral signaling and the RNAi machinery in mammalian cells. *Cell Host Microbe* **14**:435–445.

Setten, R. L., Rossi, J. J. & Han, S. (2019) The current state and future directions of RNAi-based therapeutics. *Nat Rev Drug Discov* **18**:421–446.

Shi, B., Keough, E., Matter, A., Leander, K., Young, S., Carlini, E., Sachs, A. B., Tao, W., Abrams, M., Howell, B. & Sepp-Lorenzino, L. (2011) Biodistribution of small interfering RNA at the organ and cellular levels after lipid nanoparticle-mediated delivery. *J Histochem Cytochem* **59**:727–740.

Shimeld, C., Hill, T. J., Blyth, W. A. & Easty, D. L. (1990) Reactivation of latent infection and induction of recurrent herpetic eye disease in mice. *J Gen Virol* **71**:397–404.

Sima, N., Wang, W., Kong, D., Deng, D., Xu, Q., Zhou, J., Xu, G., Meng, L., Lu, Y., Wang, S. & Ma, D. (2008) RNA interference against HPV16 E7 oncogene leads to viral E6 and E7 suppression in cervical cancer cells and apoptosis via upregulation of Rb and p53. *Apoptosis* **13**:273–281.

Sioud, M. (2005) Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J Mol Biol* **348**:1079–1090.

Snead, N. M., Wu, X., Li, A., Cui, Q., Sakurai, K., Burnett, J. C. & Rossi, J. J. (2013) Molecular basis for improved gene silencing by Dicer substrate interfering RNA compared with other siRNA variants. *Nucleic Acids Res* **41**:6209–6221.

Snijder, B., Sacher, R., Rämö, P., Liberali, P., Mench, K., Wolfrum, N., Burleigh, L., Scott, C. C., Verheije, M. H., Mercer, J., Moese, S., Heger, T., Theusner, K., Jurgeit, A., Lamparter, D., Balistreri, G., Schelhaas, M., De Haan, C. A. M., Marjomäki, V. et al. (2012) Single-cell analysis of population context advances RNAi screening at multiple levels. *Mol Syst Biol* **8**:579.

Sohail, M., Doran, G., Riedemann, J., Macaulay, V. & Southern, E. M. (2003) A simple and cost-effective method for producing small interfering RNAs with high efficacy. *Nucleic Acids Res* **31**:38e.

Stein, D. A., Perry, S. T., Buck, M. D., Oehmen, C. S., Fischer, M. A., Poore, E., Smith, J. L., Lancaster, A. M., Hirsch, A. J., Slifka, M. K., Nelson, J. A., Shresta, S. & Früh, K. (2011) Inhibition of dengue virus infections in cell cultures and in AG129 mice by a small interfering RNA targeting a highly

conserved sequence. *J Virol* **85**:10154–10166.

Suhy, D. A., Kao, S. C., Mao, T., Whiteley, L., Denise, H., Souberbielle, B., Burdick, A. D., Hayes, K., Wright, J. F., Lavender, H., Roelvink, P., Kolykhalov, A., Brady, K., Moschos, S. A., Hauck, B., Zelenaia, O., Zhou, S., Scribner, C., High, K. A. et al. (2012) Safe, long-term hepatic expression of anti-HCV shRNA in a nonhuman primate model. *Mol Ther* **20**:1737–1749.

Tabernero, J., Shapiro, G. I., LoRusso, P. M., Cervantes, A., Schwartz, G. K., Weiss, G. J., Paz-Ares, L., Cho, D. C., Infante, J. R., Alsina, M., Gounder, M. M., Falzone, R., Harrop, J., White, A. C. S., Toudjarska, I., Bumcrot, D., Meyers, R. E., Hinkle, G., Svrzikapa, N. et al. (2013) First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discov* **3**:406–417.

Takimoto, K., Wakiyama, M. & Yokoyama, S. (2009) Mammalian GW182 contains multiple Argonaute-binding sites and functions in microRNA-mediated translational repression. *RNA* **15**:1078–1089.

Taylor, B. (2010) Viral Tegument. Available at: https://en.wikipedia.org/wiki/Viral_tegument (Accessed: 9 April 2021).

Tuokko, H., Bloigu, R. & Hukkanen, V. (2014) Herpes simplex virus type 1 genital herpes in young women: Current trend in Northern Finland. *Sex Transm Infect* **90**:160–160.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008) MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**:780–783.

Wald, A. & Link, K. (2002) Risk of human immunodeficiency virus infection in herpes simplex virus type 2-seropositive persons: A meta-analysis. *J Infect Dis* **185**:45–52.

Ward, P. L. & Roizman, B. (1994) Herpes simplex genes: The blueprint of a succesful human pathogen. *Trends Genet* **10**:267–274.

Watanabe, T., Sudoh, M., Miyagishi, M., Akashi, H., Arai, M., Inoue, K., Taira, K., Yoshiba, M. & Kohara, M. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther* **13**:883–892.

Whiley, D. M. & Sloots, T. P. (2005) Sequence variation in primer targets affects the accuracy of viral quantitative PCR. *J Clin Virol* **34**:104–107.

Whitley, R. J., Kimberlin, D. W. & Roizman, B. (1998) Herpes simplex viruses. *Clin Infect Dis* **26**:541–553.

Whitley, R. J. & Roizman, B. (2001) Herpes simplex virus infections. *Lancet* **357**:1513–1518.

Whitley, R. J. & Roizman, B. (2016) Herpes simplex viruses. In: Richman, D., Whitley, R., Hayden, F. (ed), Clinical virology, 4th edition, pp. 415–445. ASM Press, Washington, DC.

WHO (2020) Billions worldwide living with herpes. Available at: https://www.who.int/news-room/detail/01-05-2020-billions-worldwide-living-with-herpes (Accessed: 14 July 2020).

Widener, R. W. & Whitley, R. J. (2014) Herpes simplex virus. *Handb Clin Neurol* **123**:251–63.

Wilhelmus, K. R., Beck, R. W., Moke, P. S., Dawson, C. R., Barron, B. A., Jones, D. B., Kaufman, H. E., Kurinij, N., Stulting, R. D., Sugar, J., Cohen, E. J., Hyndiuk, R. A. & Asbell, P. A. (1998) Acyclovir for the prevention of recurrent herpes simplex virus eye disease. *N Engl J Med* **339**:300–306.

Wittrup, A., Ai, A., Liu, X., Hamar, P., Trifonova, R., Charisse, K., Manoharan, M., Kirchhausen, T. & Lieberman, J. (2015) Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat Biotechnol* **33**:870–876.

Wittrup, A. & Lieberman, J. (2015) Knocking down disease: A progress report on siRNA therapeutics. *Nat Rev Genet* **16**:543–552.

Wolfrum, C., Shi, S., Jayaprakash, K. N., Jayaraman, M., Wang, G., Pandey, R. K., Rajeev, K. G., Nakayama, T., Charrise, K., Ndungo, E. M., Zimmermann, T., Koteliansky, V., Manoharan, M. & Stoffel, M. (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotechnol* **25**:1149–1157.

Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**:3011–3016.

Yu, B., Zhao, X., Lee, L. J. & Lee, R. J. (2009) Targeted Delivery Systems for Oligonucleotide Therapeutics. *AAPS J* **11**:195–203.

Yuen, M. F., Schiefke, I., Yoon, J. H., Ahn, S. H., Heo, J., Kim, J. H., Lik Yuen Chan, H., Yoon, K. T., Klinker, H., Manns, M., Petersen, J., Schluep, T., Hamilton, J., Given, B. D., Ferrari, C., Lai, C. L., Locarnini, S. A. & Gish, R. G. (2020) RNA interference therapy with ARC-520 results in prolonged hepatitis B surface antigen response in patients with chronic hepatitis B infection. *Hepatology* **72**:19–31.

Zamora, M. R., Budev, M., Rolfe, M., Gottlieb, J., Humar, A., DeVincenzo, J., Vaishnaw, A., Cehelsky, J., Albert, G., Nochur, S., Gollob, J. A. & Glanville, A. R. (2011) RNA interference therapy in lung transplant patients infected with respiratory syncytial virus. *Am J Respir Crit Care Med* **183**:531–538.

Zhang, X. & Qu, F. (2014) Plant virus silencing suppressors and RNA silencing in plants. In: John Wiley & Sons, Ltd (ed), eLS. Chichester, UK.

Zhou, H., Jin, M., Yu, Z., Xu, X., Peng, Y., Wu, H., Liu, J., Liu, H., Cao, S. & Chen, H. (2007) Effective small interfering RNAs targeting matrix and nucleocapsid protein gene inhibit influenza A virus replication in cells and mice. *Antiviral Res* **76**:186–193.

Zuckerman, J. E., Choi, C. H. J., Han, H. & Davis, M. E. (2012) Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. *Proc Natl Acad Sci U S A* **109**:3137–3142.