Treatment of herpes simplex virus infection using antiviral siRNA swarms with 2′-fluoro-modifications

Master's Thesis

University of Turku

MSc Degree Programme in Biomedical Sciences

Drug Discovery and Development

4/2020

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KALKE, KIIRA: Treatment of herpes simplex virus infection using antiviral siRNA swarms with 2'-fluoro-modifications
Master's Thesis, 94 p
MSc Degree Programme in Biomedical Sciences, Drug Discovery and Development
April 2020

Herpes simplex virus 1 (HSV-1) is a very common pathogen. Besides mostly harmless oral lesions, HSV-1 causes severe diseases such as neonatal herpes, herpes encephalitis and herpes keratitis, the primary cause of infectious blindness worldwide. The available anti-herpes chemotherapy is efficient but depends on a functional viral thymidine kinase. Long-term treatment, required especially in severe diseases, promotes emergence of thymidine kinase mutant strains. These strains are multi-drug resistant, and may lead to dangerous untreatable exacerbations, demonstrating an evident unmet medical need.

Small interfering RNA (siRNA) swarms are a novel antiviral approach with extensive tolerance for pathogen mutations. In contrast to regular siRNAs targeting around twenty nucleotides, swarms can target thousands, and thus overcome major challenges of regular antiviral-siRNAs, such as emergence of resistant mutant strains. The most extensively studied siRNA swarm target is the essential UL29 gene of HSV-1. The UL29 targeting siRNA swarm has proven antiviral efficacy against multiple patient-derived strains *in vitro* and significant inhibition of virus replication *in vivo*. Here, the swarm is improved by 2'-fluoro-modifications to achieve advanced stability and potency. In this Master's thesis, effects of incorporated 2'-fluoro-nucleotides on cellular tolerability, host responses and antiviral efficacy are studied *in vitro*.

According to the results, the modified siRNA swarms are well tolerated and demonstrate high antiviral efficacy in prophylactic and therapeutic settings *in vitro*. The modified siRNA swarms were better than or equal to the nonmodified siRNA swarms in every studied aspect. Overall, the results encourage for subsequent *in vivo* experiments utilizing the modified siRNA swarms.

Keywords: Herpes Simplex, siRNA, Antiviral, RNA interference

TURUN YLIOPISTO
Biolääketieteen laitos, Lääketieteellinen tiedekunta

KALKE, KIIRA: Herpes simplex -infektion hoito 2'-fluoromuunnelluilla siRNA-parvilla
Pro Gradu -tutkielma, 94 s
MSc Degree Programme in Biomedical Sciences, Drug Discovery and Development
Huhtikuu 2020

Herpes simplex virus tyyppi 1 (HSV-1) on yleinen taudinaiheuttaja, joka tunnetaan parhaiten aiheuttamistaan epämiellyttävistä ja toistuvista yskänrokoista. HSV-1 voi kuitenkin aiheuttaa myös vakavampia tautitiloja, kuten sarveiskalvontulehdusta. HSV-1:n aiheuttama sarveiskalvontulehdus on maailman yleisin sokeuteen johtava infektioperäinen sairaus, johon nykyinen lääkehoito on riittämätön.
Herpesinfektioiden nykyinen lääkehoito on tehokasta, mutta edellyttää viruksen oman tymidiinikinaasigeenin toimintaa. Erityisesti vakavammissa sairauksissa vaadittava pitkäaikainen ja ennaltaehkäisevä lääkehoito voi johtaa tymidiinikinaasimutatoituneiden viruskantojen ilmaantumiseen. Nämä lääkeresistentit viruskannat ovat selkeä puute nykyisessä lääkehoidossa, ja siten tärkeä lääkekehityskohde.

Pienet häiritsevät RNA:t eli siRNA:t (*engl. small interfering RNA*) johtavat geenien hiljentymiseen. Niitä voidaan käyttää estämään virusten lisääntymistä kohdistamalla siRNA:t kohdeviruksen välttämättömään geeniin. Tavallisesti siRNA:t on kohdistettu verrattain lyhyeen sekvenssijaksoon. Pieni kohdealue altistaa tehon menetykseen joko lähisukuisten virusten perimän monimuotoisuuden vuoksi tai mutaation kautta. SiRNA-parvet tuotetaan entsymaattisesti pitkästä kohdealueesta, jolloin niiden teho kattaa viruskantojen monimuotoisuuden ja mahdolliset mutaatiot kohdesekvenssissä. SiRNA-parvia on tutkittu lääkkeenä herpesinfektioon sekä *in vitro* että *in vivo* erittäin lupaavin tuloksin. SiRNA:t kuitenkin hajoavat nopeasti elimistössä, mikä vähentää hoitomuodon potentiaalia, annostelutavasta riippuen. SiRNA:n kestävyyttä pystytään kuitenkin parantamaan kemiallisilla modifikaatioilla.

Tässä Pro Gradu -tutkielmassa selvitettiin siRNA-parviin sisällytettyjen 2'-fluoro-nukleotidien vaikutusta parvien tehoon herpestä vastaan *in vitro*. Lisäksi selvitettiin solujen luonnollisen immuniteetin vaste muunnelluille siRNA-parville. Tutkimuksissa käytetyt solulinjat edustivat hermostoa ja sarveiskalvoa, jotka ovat olennaisia herpesinfektion kohdekudoksia. Muunnellut siRNA-parvet ovat tulosten perusteella vähintään yhtä turvallisia ja jopa tehokkaampia kuin perinteiset siRNA-parvet. Molempien parvityyppien havaittiin lisäksi estävän virusinfektiota ainakin viiden vuorokauden ajan kerta-annostelun jälkeen. Tulokset valottavat perinteisten ja erityisesti muunneltujen siRNA-parvien potentiaalia terapeuttisena ja ennaltaehkäisevänä lääkehoitomuotona ja kannustavat niiden jatkotutkimuksiin *in vivo*.

Avainsanat: Herpes simplex, siRNA, RNA-interferenssi, Virustautien lääkehoito

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1 Introduction

1.1 Human herpesviruses

The herpesviruses (*Herpesviri*dae) are divided into alpha-, beta- and gamma herpesviruses based on their nucleotide (nt) sequence and biological characteristics. The herpesviruses share many common features with each other, such as their structure (see **Figure 1**). In general, herpesviruses are large, enveloped viruses (120-300 nm), with a double-stranded DNA protected by an icosahedral capsid. They also have the capability to establish latency after primary infection, which means that they can persist long time periods in the host before reactivating. (Cann, 2016)

Altogether there are over 100 different herpesviruses, of which humans carry nine (see **Table 1**). In addition to the nine human herpesviruses (HHV), the B virus (*Cercopithecine Herpesvirus 1*) of macaques is capable of infecting humans and causing fatal encephalitis (Cohen et al., 2002).

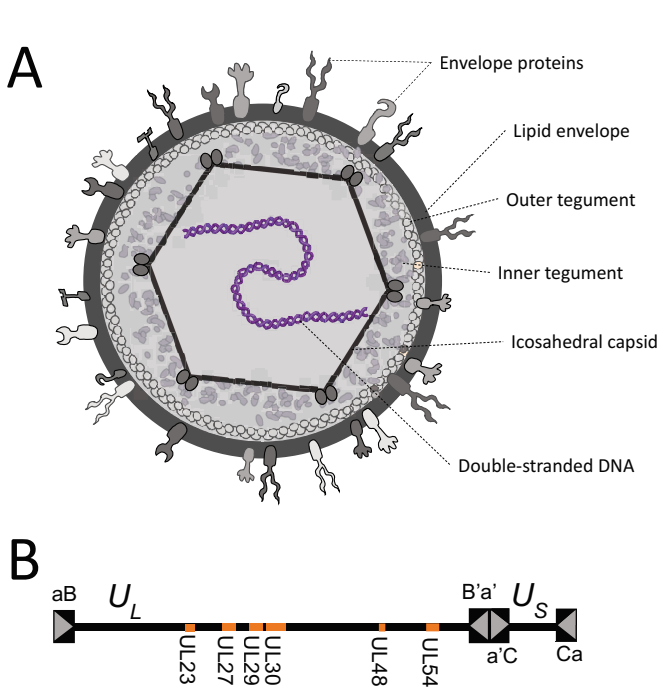

Figure 1 - Structure of herpes virion and herpes simplex virus genome. A) The double-stranded DNA genome of the virus is protected by an icosahedral capsid. Outside the capsid, are the inner and outer teguments, containing essential proteins for viral lifecycle. On the outer surface of the virion, the envelope embedded with various glycoproteins guide the attachment of the virus to the host cell. **B)** The genome of herpes simplex viruses consists of two unique regions, the long (U_L) and short (U_S) DNA segment. The U_L is flanked by repeat sequences B and inverted B (B'), whereas U_S is flanked by C and C'. Shorter repeat sequences (a and a') surround the segments. The repeats enable four conformations of the genome. Selected relevant genes of herpes simplex virus 1 are highlighted on the genomic map. The genome structure graphics is modified from (Paavilainen, 2017).

In general, HHVs are relatively common viruses. Their seroprevalence (proportion of the viral antibody positive within a population, %) is overall higher in developing countries, but differentiates depending on more exact geographical location and socioeconomic status. As an example, in a study conducted in USA, pregnant women below poverty level had herpes simplex virus 1 (HSV-1) seroprevalence of 71.8-84.5% compared to 47.5-58.9% of the women above poverty level (Patton et al., 2018). Furthermore, the herpesviruses are more dangerous for immunodeficient patients who are more likely to develop severe diseases (e.g. HSV-1), or symptoms at all (e.g. HCMV). To this date, the only herpesvirus against which a vaccine is available, is the varizella-zoster virus (VZV) (Varivax®, Zostavax®). For this Master's thesis, the most important herpesvirus is herpes simplex virus 1 (HSV-1), on which the following chapters will focus.

Table 1 – Human herpesviruses and common diseases caused. Modified from (Paavilainen, 2017).

	HHV	Common name	Common diseases or symptoms
Alpha-herpesviruses	1	Herpes simplex virus 1 (HSV-1)	Orofacial and genital lesions
	2	Herpes simplex virus 2 (HSV-2)	Genital lesions
	3	Varizella-zoster virus (VZV)	Chickenpox (primary infection) and shingles (reactivation)
Beta-herpesviruses	4	Epstein-Barr Virus (EBV)	Mononucleosis, also associated with malignancies
	5	Cytomegalovirus (HCMV)	Often asymptomatic, light fever, mononucleosis
	6A	Human herpesvirus 6 A (HHV-6A)	Roseola
	6B	Human herpesvirus 6B (HHV-6B)	Exanthema subitum (Roseola infantum), Fever
Gamma-herpesviruses	7	Human herpesvirus 7 (HHV-7)	
	8	Kaposi's sarcoma-associated herpes virus (KSHV, HHV-8)	Prerequisite for Kaposi's Sarcoma

1.2 Herpes simplex virus 1

HSV-1 is evolutionally very old dating back almost six million years (Norberg et al., 2011; Wertheim et al., 2014). Throughout its evolution, it has adapted to its viral life with humans. Its smart ways of adaptation can be seen in its ability to modify the host immune system to its liking, and the very effective latency-reactivation way of viral life – to this date, HSV-1 infection cannot be entirely cured, nor prevented by vaccination.

In this chapter, the structure, viral lifecycle and related gene regulation, as well as host responses to HSV-1 are introduced. In the following chapters, the most important morbidities caused by HSV-1 to humans are presented followed by chapters reviewing current as well as potential future treatment approaches against HSV-1.

1.2.1 Structure of HSV-1 virion

The size of the HSV-1 virion is roughly 225 nm. Without taking the surface glycoproteins into account, the size ranges from 170-200 nm (Grünewald et al., 2003). The outer surface of the virion consists of the lipid envelope derived from host cell membrane. In the envelope, there are embedded viral proteins such as glycoproteins. The most abundant envelope protein is glycoprotein B (gB), which similarly to other glycoproteins, mediates cellular entry of the virus (Cai et al., 1988). Under the envelope lie the outer and inner teguments, in that order. The tegument contains over 20 viral proteins (Loret et al., 2008) with multiple important functions related for example to initiation of viral transcription and regulation of the host cell's immune response. Inside the tegument is the capsid, which protects the DNA. The HSV-1 capsid consists of four viral proteins forming pentons, hexons and triplexes, of which twenty come together to form a sphere-like icosahedron referred as the icosahedral capsid (Yuan et al., 2018; Zhou et al., 2000).

Inside the capsid is the linear, double stranded DNA genome, which has approximately 152,000 base pairs and encodes over 80 genes, which map to both genomic strands and can overlap. The genome consists of two larger regions, named as the unique short (U_S) and unique long (U_L) -regions, surrounded by long

inverted repeat sequences at both ends of the regions. The genome has four different conformations (Hayward et al., 1975). For an illustration of the herpes virion, please see **Figure 1A**. For illustration of the viral genome organization and location of selected HSV genes in the genome, please see **Figure 1B**.

Additionally, the virion contains multiple cellular proteins (Loret et al., 2008). Interestingly, RNA-mediated silencing of these proteins led to poorer infectibility of the viruses, suggesting their importance in the viral lifecycle (Stegen et al., 2013).

1.2.2 Lifecycle of HSV-1

HSV-1 can transmit from human to human via secretions, such as saliva, or from the clinical lesions resulting from viral activity. The primary infection establishes through physical or chemical breaks in the epithelia. Commonly, the lytic, primary infection in the epithelial area of entry is clinically mild or even asymptomatic (Petti and Lodi, 2019).

The virus enters the cell by fusion of the viral envelope to the cell membrane, or via endocytosis, after attachment to the cell surface (please see **Figure 2A**). The attachment and fusion of HSV-1 are complex. In short, the viral glycoprotein C (gC) first attaches to heparan sulphate proteoglycans (HSPG) of the cell. After attachment, gD initiates the entry by interaction with cellular receptors, such as nectin-1 or herpesvirus entry mediator (HVEM). The fusion itself is mediated by gB, gH, and gL viral glycoproteins. (Campadelli-Fiume et al., 2007)

After fusion of viral and cellular membranes, the capsid and tegument of HSV-1 are released to the cytoplasm. Through microtubule and dynein-mediated transport of the capsid, the genome is released to the nucleus (Sodeik et al., 1997). When in the nucleus, the viral gene expression initiates, and the genes are expressed in a cascade-like manner (Honess and Roizman, 1974). In the nucleus, the viral genome is replicated, new capsids are formed, and they are combined as nucleocapsids (Mettenleiter, 2002). The final assembly takes place outside of the nucleus. In short, during the route from the nucleus to the cellular membrane, the virus is primarily enveloped at the nuclear membrane, de-enveloped, tegumented and secondarily enveloped at non-nuclear specialized vesicles (Owen et al., 2015). Eventually, mature progeny virions exit the cells via exocytosis. They may then proceed to infect

neighboring cells either apically or laterally. After the replication cycle, the host cell is destroyed. Hence, the described process is called a lytic infection.

After the primary infection, the virus can hide from the host and wait for a new lytic cycle by establishing a latent infection. The establishment of latency requires that the virus breaches the nerve-blood-barrier and is transported by retrograde transport via sensory neuron axon to ganglia. In the case of HSV-1, the target is most commonly the trigeminal ganglia which is reached via the ophthalmic, maxillary, or mandibular nerve branch. In the neuronal somas, the virus hides from the host's defense systems by expressing only a certain genomic region; the latency associated transcript (LAT) (Stevens et al., 1987). The expression of LAT for example inhibits apoptosis of the host and aims to prevent the virus from reactivating. Eventually various factors, such as those leading to cellular stress (Avgousti and Weitzman, 2015; Wilson and Mohr, 2012) can reactivate HSV-1. The reactivation of HSV-1 infection can be symptomatic or asymptomatic. In both cases viral shedding and thus subsequent transmission is possible. The viral lifecycle of HSV-1 is presented in **Figure 2B**.

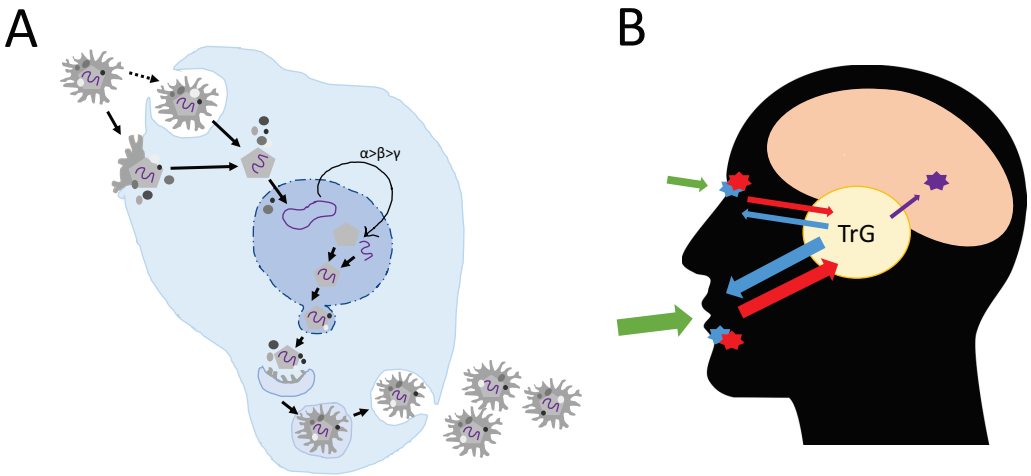

Figure 2 - Lifecycle of HSV-1. A) Replication of HSV-1 in cell. The virus enters the cells via fusion or endocytosis. The nucleocapsid is transported to the nucleus, into which the genome is released. The transcription happens in a cascade-like manner, after which the nucleocapsid is formed in the nucleus. Outside the nucleus, the envelope, its glycoproteins and the tegument are assembled, and the mature virions exit the cells. **B)** After the primary lytic infection (green arrows), the virus can transfer by retrograde transport (red arrows) to the trigeminal ganglia (TrG), where it can establish latency. Upon reactivation, the virus is transferred back to the epithelium by anterograde transport (blue arrows) via trigeminal nerve branches. Lytic, clinical or subclinical infection can occur after reactivation (blue stars), or during primary infection (red stars). During reactivation or primary infection, the virus can proceed to infect the brain (purple arrow, purple star) or to the eye.

1.2.3 Regulation of gene expression

The gene expression of HSV-1 in lytic infection is regulated in a cascade-like manner. The three, coordinately expressed gene groups, are referred as α-, β-, and γ-genes, or immediate early (IE), early (E) and late (L) -genes, respectively (Honess and Roizman, 1974). In the lytic infection α-genes are expressed 2-4 hours post infection (hpi), β-genes 5-7 hpi and γ-genes latest at 12 hpi (Honess and Roizman, 1974). The expression of α-gene products is a requirement for expression of β-genes, which then again are a requirement for γ-gene expression (Honess and Roizman, 1975; Roizman et al., 1975). In general, alpha and beta genes form a negative feedback-loop, where gene products expressed later inhibit the transcription of the earlier genes. However, during the first steps of reactivation from a latent infection, the gene expression of the different gene groups seems to happen simultaneously to enable viral protein-mediated shut-down of the latent state (Linderman et al., 2017; Mattila et al., 2015).

In lytic infection, the initiation of transcription of the α-genes, and thus the initiation of the whole expression cascade requires the viral alpha-trans-inducing factor (α-TIF/VP16) that is translocated to the nucleus with the viral DNA (Pellett et al., 1985). It serves as a transcription factor for α-genes in complex with several host proteins (e.g. HCF1, Oct1, LSD1) (Kristie and Roizman, 1987; Liang et al., 2009; McKnight et al., 1987). The expression of genes other than ICP0 (infected cell polypeptide 0) also requires the recruitment of CLOCK histone acetyl transferase (Kalamvoki and Roizman, 2010).

The α-gene products include, for example, the above-mentioned ICP0 (encoded by α0 gene), which serves as a gene transactivator via enhancement of CLOCK activity (Kalamvoki and Roizman, 2010), and ICP27 (encoded by UL54), which is a regulatory protein required for all γ-gene expression, and is an inhibitor of host cell apoptosis (Aubert and Blaho, 1999). An example of β-genes, which include proteins required for replication of DNA, is ICP8 (encoded by UL29), a single-stranded DNA binding protein required for viral DNA synthesis (de Bruyn Kops and Knipe, 1988). Of the γ-genes, a majority encode for glycoproteins of the viral envelope, such as glycoprotein B (encoded by UL27) essential for virus mediated cellular entry (Cai et al., 1988), or for tegument proteins, such as α-TIF (encoded by UL48). All the above-

mentioned genes are essential for viral lifecycle of HSV-1. In addition to the essential genes, HSV-1 has many accessory proteins non-essential for viral replication.

1.2.4 Host responses to HSV-1 infection

The innate immunity responses against HSV-1 begin immediately upon the cellular entry of HSV-1. The host responses aim at eradication and destruction of the virus, and importantly also aim at preventing spread of the virus by making the surrounding cells (and tissue) alert for the potential incoming pathogen. HSV-1, however, fights back, by modifying and inhibiting the host responses to its benefit in multiple different ways (Hukkanen et al., 2010; Kurt-Jones et al., 2017). The host innate responses to HSV-1, such as type I interferons (IFN), are highly antiviral and can protect against a viral disease (Isaacs and Lindenmann, 1957; Minkovitz and Pepose, 1995). Thus, the success of the host's innate response is major factor in the clinical (and *in vitro*) outcome of the infection. Overly extensive immune response to HSV-1, however, may lead to severe or even lethal exacerbations (Lundberg et al., 2008), while too low levels of immune response during HSV-1 infection, such as those of immunocompromised individuals, may as well lead to severe outcomes (WHO, 2017).

HSV-1 is detected in cells by multiple pattern recognition receptors (PRR), which can detect pathogen associated molecular patterns (PAMPs), such as viral proteins or viral nucleic acids, and damage-associated molecular patterns (DAMPs). The innate PRRs induce the production of cytokines, chemokines and type I IFNs, which subsequently induce multiple IFN stimulated genes (ISGs). Examples of ISGs are for example human myxovirus resistant protein 2 (MxB), which inhibits the HSV-1 genome delivery to the nucleus (Crameri et al., 2018) and human myxovirus resistance protein 1 (MxA), which reduces the replication potency of HSV-1 (Ku et al., 2011). HSV-1 can however alter the actions MxA to support the infection (Ku et al., 2011). These responses mentioned above, and their magnitudes may vary from one cell type to another, however, the general idea persists.

Toll-like receptors (TLRs) are important PRRs in the host for recognition of HSV-1. In general, they induce production of proinflammatory cytokines (TLR2) or induce type I IFN response (TLR3/7/8/9). The cytokine production happens mostly via MyD88 (myeloid differentiation primary response 88) and NF-κB (nuclear factor

kappa-light-chain-enhancer of activated B cells), whereas the type I IFN response happens mostly via IRF3 (interferon regulatory factor 3). The IRF3 pathway is inhibited at least by VP16 (Xing et al., 2013) and ICP34.5 (Verpooten et al., 2009). The actions of IRF3 happen via phosphorylation, dimerization and subsequent translocation to the nucleus, where it interacts with the IFN promoter and thus induces type I IFN (IFN-α, IFN-β) expression. The cellular IFN expression then induces expression of ISGs via the Jak/Stat pathway, which is, then again, inhibited by viral VHS (virion host shutoff protein). (Jahanban-Esfahlan et al., 2019; Kurt-Jones et al., 2017)

Upon cellular entry HSV-1 is detected by the cell by TLR2, which recognizes the viral glycoproteins of the virion surface (Kurt-Jones et al., 2004). The effects of TLR2 are however inhibited by viral protein ICP0, which subsequently inhibits NF-κB induction (van Lint et al., 2010). In the cell TLR3, TLR7, TLR8, and TLR9, which are all located on endosomal surfaces, can detect viral nucleic acids. TLR7 and TLR8 sense single stranded RNA (ssRNA), whereas TLR3 detects dsRNA (Jahanban-Esfahlan et al., 2019). The viral gene US3 is known to control the TLR3 responses, and thus also reduce type I IFN and ISG induction (Peri et al., 2008).

In addition to the TLRs, many other PRRs detect viral RNA as well. The cytosolic RIG-I (retinoic acid-induced gene I) recognizes ssRNA (related to RNA polymerase III activity), and eventually induces type I IFN responses (Chiu et al., 2009). Other important PRRs that recognize viral dsRNA, are RIG-I-like receptors, such as MDA5 (melanoma differentiation-associated protein 5) (Melchjorsen et al., 2010) and PKR (protein kinase R). The antiviral activity of PKR is related to its ability to inhibit translation and promote antiviral autophagy (Kurt-Jones et al., 2017), yet these actions of PKR are inhibited by the viral ICP34.5 (He et al., 1997; Orvedahl et al., 2007).

HSV-1 can also be detected in cells by its DNA. Of the TLRs, TLR9 detects genomic DNA motifs of HSV-1 (Krug et al., 2004; Lund et al., 2003). Additional important DNA sensing PRRs are cGAS (cyclic GMP-AMP synthase) and IFI16 (gamma-interferon-inducible protein 16). The cytosolic cGAS induces STING (stimulator of interferon genes), which in turn indirectly activates IRF3 (Li et al., 2013; Sun et al., 2013). The activity of STING is nevertheless prevented by viral ICP27 (Christensen et al., 2016). The IFI-16 also works via the IRF3 pathway. In addition to cytosolic viral DNA

recognizing activity, IFI-16 can also silence viral genes epigenetically in the nucleus as well as induce IRF3 signaling from the nucleus (Johnson et al., 2014; Orzalli et al., 2012). The viral protein ICP0 can however degrade IFI-16, thus limiting also its epigenetic silencing functions (Orzalli et al., 2012).

The mentioned PRRs and the innate responses they eventually induce were only a brief review on the subject. Likewise, the innate immunity evasion methods of HSV-1 are more numerous. Importantly, the immune evasion can also target the adaptive immune system. As important examples of adaptive immune evasion, HSV-1 can disable recognition of the infected cells by preventing presentation of viral antigens on the cellular surface by preventing actions of major histocompatibility complex (MHC) class I (Früh et al., 1995; Hill et al., 1995) and MHC class II (Neumann et al., 2003), as well as by preventing antibody mediated antiviral response by binding IgG (Dubin et al., 1991) and by preventing complement activation (Friedman et al., 1984). Additionally, the lack of an HSV vaccine can be considered as indirect evidence of the competence of HSV-1 to evade the adaptive immune system. A common understanding of the interactions between the virus and the host are a prerequisite for development of antiviral drugs, especially those with possible interactions with the same pathways by which the virus is detected by.

1.3 Herpes simplex virus 1 – a common pathogen

Primarily, HSV-1 is known for infecting the orofacial area, whereas its closest relative, herpes simplex virus 2 (HSV-2) is considered to infect the genital area. The most common symptoms are sores or blisters in the orolabial area, before which, the patient will experience tingling or itching (WHO, 2017). The clinical manifestations of reactivations are most commonly labial cold sores, but can also be lesions on nose, chin or cheek skin, which last for 2-16 days (Petti and Lodi, 2019).

Overall, HSV-1 is a highly common pathogen. In 2012 the world-wide prevalence of HSV-1 was 67%, which is roughly 4.9 billion people (Looker et al., 2015a). In Finland, however, the number of HSV-1 seropositive people has decreased from the beginning of the ‘90s. For example, the seroprevalence of HSV-1 in pregnant women has decreased significantly from 1992 to 2012 by almost 25% (from 69.5% to 45%), and the corresponding seroprevalence of HSV-2 by 6.5% (from 17.5% to 11%) (Puhakka et al., 2016). The decrease in seroprevalence increases the risk of primary infections at later age, for example during pregnancy, which may cause the severe neonatal HSV infection. In addition, immunocompromised patients are especially at risk of the severe herpes simplex complications, such as pneumonia, disseminated infections and hepatitis, as well as having an increased risk for herpes keratitis, the leading cause of infectious blindness worldwide. However, HSV-1 can also cause severe infections in immunocompetent hosts. The more severe morbidities caused by HSV-1 are introduced in the following chapters. Notably, these potentially fatal morbidities, such as encephalitis, are not favorable for HSV-1 either, since a deceased host is not as beneficial for the virus as a viable host.

1.3.1 Genital herpes

Even though HSV-2 is usually considered to cause genital herpes lesions, and HSV-1 orofacial lesions, HSV-1 has gained more importance as a causative agent of genital herpes. Recently, in Finland, HSV-1 has become the main cause for genital herpes among young women (Tuokko et al., 2014), regardless of the decrease in HSV-1 seroprevalence (Kortekangas-Savolainen et al., 2014). Worldwide, in 2012, the number of people with genital HSV-1 was 140 million (Looker et al., 2015a) in contrast to 417 million people with genital HSV-2 (Looker et al., 2015b).

The genital HSV-1 can be asymptomatic or symptomatic. When it is symptomatic, painful ulcers or blisters, or both, occur in the genital area. Moreover, at least pre-existing HSV-2 infection can make the patient more susceptible to human immunodeficiency virus 1 (HIV-1) infection (Desai and Kulkarni, 2015; WHO, 2017). However, prior HSV-1 genital infection can protect a patient from symptomatic genital HSV-2 infection (Hofstetter, Rosenthal and Stanberry 2014).

In times of active clinical symptoms, shedding of HSV-1 is most abundant, and thus is also most contagious, however, subclinical, asymptomatic shedding also enables virus transmission (Barton et al., 1987). The frequency of subclinical HSV-1 shedding is up to 2.8% of asymptomatic days, yet is highest after symptomatic HSV-1 lesions (Wald et al., 1995). According to more recent studies 2.2%-2.8% of oral DNA samples are HSV positive at any given time (Mäki et al., 2015; Mäki et al., 2018). Overall, the recrudescence of symptomatic genital HSV-1 are rarer than that of HSV-2, taking place approximately once compared to four times per year (Hofstetter et al., 2014). Epidemiologically, the frequent, asymptomatic reactivations of genital HSV-1, as well as asymptomatic oral HSV-1 reactivations, might have significant effects to public health since they can lead to unforeseen transmission via genital or oral-genital contact. Hence, there is a need for an antiviral drug, preventing viral shedding, or even better, reactivations altogether.

1.3.2 Neonatal herpes simplex infection

Neonatal herpes is a complication of genital herpes resulting from HSV-1 or HSV-2. The overall rate of neonatal herpes is 14,000 cases per year worldwide. In Europe, 1000 cases arise per year, of which 57% result from HSV-1. However, globally HSV-2 is the major cause of neonatal herpes infections (70% of cases). (Looker et al., 2017)

Neonatal herpes infection occurs when HSV is transmitted from the mother to the infant. The transmission may happen *in utero, postpartum* or, most commonly (85%) during childbirth (James and Kimberlin, 2015). The transmission can be prevented by cesarean delivery, or by avoiding invasive monitors during child birth, or at best, preventing maternal infection altogether (Brown et al., 2003). Overall, the highest risk for transmission from mother to infant is when the mother has primary HSV infection (Brown et al., 2003). Alarmingly, since the proportion of HSV seropositive

women has decreased, the risk for primary HSV infection during pregnancy has increased, which subsequently creates an elevated risk for neonatal herpes infection. Fortunately, the incidence of neonatal herpes has remained unchanged so far (Puhakka et al., 2016).

Almost half of the infants infected during or after childbirth will develop central nervous system (CNS) symptoms. In these situations, the mortality with antiviral treatment is 4% after one year, and two thirds of the survivors develop neurodevelopmental outcomes. When the infection is disseminated into other organs, such as liver or lungs, the mortality is close to 30%. In the rare *in utero* cases, the clinical manifestations include e.g. microcephaly, skin lesions or ocular findings, which are present already at birth. Luckily, close to half of all of the cases of neonatal herpes do not involve CNS or organ system symptoms. (James and Kimberlin, 2015)

1.3.3 Herpes simplex encephalitis

HSV-1 is the most common cause of sporadic, infectious encephalitis. The caused herpes simplex encephalitis (HSE) is at worst a hemorrhagic and necrotizing condition in the frontal and temporal lobes of the brain (Kumar et al., 2013). Approximately a third of HSE cases result from primary infection (Whitley, 2006), but the majority happen due to reactivations (**Figure 2B**). The adverse access of HSV to the brain may result for example as consequence of enhanced neurotropism of reactivating virus, lacking antiviral activity of the patient, or overall high viral load. For the establishment of HSE, at least three routes are possible; through the olfactory nerve route or through hematogenous or the trigeminal pathway (Petti and Lodi, 2019). The disease has an incidence of 1 in 200,000-500,000 per year in developed countries and as high as 30% mortality rate even with proper treatment (Whitley, 2006). The disease affects mostly children and young adults but can affect any age group (Kumar et al., 2013). Most notably, immunocompetent individuals actually have an equal risk of HSE compared to immunocompromised individuals (Whitley, 2006).

1.3.4 Herpes simplex keratitis

HSV-1 is linked to many ocular diseases with manifestations of conjunctivitis, acute retinal necrosis, and epithelial and stromal keratitis, which together are referred as herpes simplex keratitis (HSK). The worldwide incidence of HSK is as high as 1.5 million with 40,000 annual cases of blindness or severe vision impairment, as categorized by the World Health Organization (Farooq and Shukla, 2012). Overall, in developed countries, HSK is the leading cause for infectious blindness (Dawson and Togni, 1976) and severe visual impairment (Farooq and Shukla, 2012). In these nations, the incidence is close to 250,000 cases per year, of which 1.5% lead to the severe visual impairments, or blindness (Farooq and Shukla, 2012).

HSK may result from primary infection of the cornea but can also occur by reactivation of latent virus of oral origin (**Figure 2B**). Labetoulle et al. (2000) detected this phenomenon *in vivo* in mice, being able to demonstrate viral reactivation to the eye on the same side of the primary labial infection (Labetoulle et al., 2000). Later, they published the possible neuronal connections leading to virus propagation from the labia to the eye in mice (Labetoulle et al., 2003). The connections explained why reactivations to the cornea are most common, compared to viral manifestations on the iris or retina, for example.

After the first clinical manifestation in the cornea, the recurrences of HSK are very likely (Dawson and Togni, 1976). For this reason, the patients use continuous prophylactic acyclovir treatment to prevent the recurrences. However, the prophylactic treatment with acyclovir induces acyclovir-resistant virus strains to emerge (Duan et al., 2009; van Velzen et al., 2013). Additionally, patient-derived virus isolates that are acyclovir-resistant, can be resistant simultaneously to other anti-herpetic drugs as well, even if the drug has totally different mechanism of action (Duan et al., 2008). Hence, emergence of multi-drug resistant viral strains during long-term prophylactic treatment encourages for development of antivirals with novel MOAs for treatment of herpes keratitis.

1.4 Treatment of herpes simplex virus 1 infections

The by far most widely used antiviral drug against HSV-1 is a guanosine analogue, acyclovir (ACV). It is the treatment of choice for all the severe and more common conditions described in the previous chapter. ACV is well tolerated and can be used orally, topically or intravenously, which is required for the most severe exacerbations, such as HSE. In general, ACV decreases the time of active symptoms and related viral shedding in therapeutic use, as well as prolongs the time between recurrences when used prophylactically. ACV has relatively low half-life of three hours and a relatively low oral bioavailability (15-30%). However, it's commonly used prodrug, valacyclovir, has better pharmacokinetics upon oral administration. (Koulu and Mervaala, 2013). The antiviral drugs approved for treatment of HSV-1 are summarized in **Table 2**.

The mechanism of action of ACV is based on the activity of viral thymidine kinase (tk) and viral DNA polymerase. In infected cells, tk phosphorylates ACV into acyclovir monophosphate, which is subsequently phosphorylated to acyclovir triphosphate by cellular kinases. In its triphosphate form, ACV competitively inhibits the activity of the viral DNA polymerase and prevents further synthesis of the viral DNA chain. This MOA is shared not only with acyclovir and valacyclovir, but also with penciclovir and its prodrug famciclovir, which have higher oral bioavailabilities and longer half-lives in comparison to ACV. And as famciclovir metabolizes to penciclovir only after first-pass metabolism, it has a much higher bioavailability in comparison to penciclovir. These afore-mentioned drugs are very effective and represent the relevant treatment against HSV-1. Due to their same mechanism of action, acyclovir-resistant strains are resistant to all the other mentioned drugs as well. Hence, in resistant patients a drug with a different MOA is required, and foscarnet is used. Foscarnet, however, has severe adverse effects, including high renal toxicity. For this reason, it is used primarily in treatment of ACV-resistant strains. Its MOA is to compete of the pyrophosphate binding site on the viral DNA-polymerase, thus competitively inhibiting its actions. (Cann, 2016; Koulu and Mervaala, 2013)

The problem with the current treatments is that the drugs used against HSV-1 share the same MOAs and target proteins (tk and viral DNA polymerase), which leads to issues with emerging resistant strains, especially in long-term prophylactic use.

Notably, due to the latent, reactivating nature of HSV-1 infection, it cannot yet be cured. These factors together lead to a need for a drug treating and/or preventing HSV with a novel MOA.

Table 2 - Current treatment of HSV-1. Modified from (De Clercq and Li, 2016).

	Structure	Mechanism of action	Administration route[a]	Indication of use[a]
Acyclovir	Guanosine analogue	Activation by viral thymidine kinase and subsequent inhibition of viral DNA polymerase	Oral, topical, intravenous[b]	Oro-facial, genital herpes, herpes keratitis, herpes encephalitis[b]
Valacyclovir			Oral	Genital and oro-facial herpes
Penciclovir			Topical	Oro-facial herpes
Famciclovir			Oral	Genital and orofacial herpes
Foscarnet	Pyrophosphate analogue	Inhibits viral DNA polymerase	Intravenous	Acyclovir-resistant herpes infection [b]
Idoxuridine	2'-deoxyuridine analogue	Substitutes thymidine, targets DNA polymerase	Topical	Herpes keratitis
Trifluridine		Inhibits DNA replication		
Brivudine		Inhibits DNA synthesis		
Docosanol	Saturated fatty alcohol	Inhibits viral fusion to cellular membrane	Topical	Oro-facial herpes
Cidofovir [c]	Nucleoside phosphonate analogue	Inhibits DNA synthesis [a]	Intravenous [a]	Acyclovir- and foscarnet-resistant herpes infection [c]

[a](DrugBank.ca, 2020), [b](Koulu and Mervaala, 2013), [c] (Blot et al., 2000)

1.4.1 Genetic variation of circulating HSV-1 strains

Clinical circulating strains of HSV-1 vary from each other by their phenotype *in vitro* and by their sequence (Bowen et al., 2019; Szpara et al., 2010). The sequence, and the phylogenic location of the strain, is believed to also influence the clinical characteristics of the infection, such as reactivation frequency (Shipley et al., 2018). Infected humans from a certain geographic location tend to have similarity in the genomics of their HSV-1 strains (Bowen et al., 2019; Pfaff et al., 2016). Overall, some genes are more conserved than others (Bowen et al. 2019). Notably, circulating viral strains, and *in vitro* reference strains, are expressing not just one genotype, but demonstrate intrastrain variability (Parsons et al., 2015; Shipley et al., 2018). Hence, within a strain, partial drug resistance might occur due to mutation in the drug target of a viral subpopulation. For example, circulating virus strains are not fully resistant or fully sensitive to acyclovir, but manifest ranging susceptibilities (Bowen et al., 2019).

1.4.2 Acyclovir resistance

A vast majority of the mutations leading to ACV resistance are in the gene encoding for thymidine kinase (UL23), but can also be found from the gene encoding for DNA polymerase (UL30) (Sauerbrei et al., 2011). The UL23 and UL30 genes have high genetic variability among strains of HSV-1, and the possible mutations are many (Schmidt et al., 2015). Most of the mutations leading to ACV resistance are found from active or conserved genomic sites, but can also be found outside of these regions (Sauerbrei et al., 2011; Schmidt et al., 2015).

Selective forces, such as long-term use of ACV, might lead to the emergence of ACV-resistant escape mutants. A very important example is herpes keratitis, which is treated prophylactically with ACV to prevent dangerous exacerbations. Adversely, the prophylaxis with ACV predisposes for ACV-resistant keratitis, and causes ACV-resistant viruses to emerge (van Velzen et al., 2013). In a study by Duan et al. (2008) they found that 6.4% of the clinical HSV-1 strains derived from immunocompetent herpes keratitis patients were ACV-resistant, whereas otherwise only 0.1-0.7% of circulating HSV-1 strains of immunocompetent patients display ACV resistance (Farooq and Shukla, 2012). Moreover, the reactivating virus strains of keratitis

patients are usually ACV-resistant and can cause sequentially multiple recurrences (Duan et al., 2009).

In addition to immunocompetent patients with long-term prophylactic treatment periods, immunocompromised patients are at high risk of developing ACV-resistant strains. However, the cause of immunosuppression affects the risk of developing ACV resistance. For example, according to a study with 1132 immunocompromised patients with HSV-1, the overall prevalence of ACV resistance was 3.1%, whereas bone marrow transplant patients had a prevalence of 12.1% (Danve-Szatanek et al., 2004).

The ACV-resistant strains are generally responsive to foscarnet (Sauerbrei et al., 2011); nevertheless simultaneously ACV- and foscarnet-resistant strains have been described in immunocompromised (Darville et al., 1998) and immunocompetent patients (Duan et al., 2008). Alarmingly, in a study by Danve-Szatenek et al. (2004), over half of bone marrow transplant patients with ACV resistance demonstrated resistance also against foscarnet (Danve-Szatanek et al., 2004). Such ACV- and foscarnet-resistant viral strains can be treated with cidofovir (Blot et al., 2000), which is normally used for treatment of CMV in immunocompromised patients. Cidofovir, however, has dangerous adverse effects, such as high renal toxicity, and is thus used only when other treatments fail, and treatment is necessary.

1.4.3 Current clinical pipeline

Majority of the current clinical pipeline for treatment of HSV-1 is focused on improved treatment of oro-facial herpes episodes, rather than treatment of the severe conditions, or preventing recurrences. The investigative, HSV-specific drugs in clinical trials listed in Clinicaltrials.gov after 2010 are presented in **Table *3***. Additionally, vaccine development and immunomodulatory drugs in the clinical pipeline are presented in the following chapters. The immunomodulatory drugs are also included in **Table *3***.

Three investigative drugs in the pipeline have unclear mechanisms of actions based on available literature and were thus left out of **Table *3***. Such drugs are 1) a topical short synthetic peptide, ZEP-3 (phase II; NCT02483182), a chemically modified version of snake venom derived short peptide described to have analgesic and antiviral purposes (SIS Shulov Innovative Science, 2018), 2) sublingual thiomersal

BTL-TML-HSV (phase II; NCT01308424), which is described to affect by inhibiting transcription and recruiting leucocytes (Beech Tree Labs Inc, 2020), and 3) NB-001 (phase III; NCT01321359), a topical nanoemulsion, with multiple ingredients including such disrupting the viral envelope (Kircik et al., 2012). None of these treatments strike as HSV-specific, however at least NB-001 is described to have specific antiviral activity unrelated to immunomodulatory functions.

Table 3 - Pipeline for treatment of HSV-1. Representing year 2010 and onwards. The HSV-1 specific investigational drugs in clinical trials are enlisted in the upper section, whereas immunomodulatory treatments are enlisted in the lower section.

Indication	Drug	MOA	Phase	Clinicaltrials.gov identifier	
Labial, facial or genital herpes simplex episodes	Amenamevir (ASP2151), Oral	Inhibition of viral helicase-primase	III	NCT01959295	HSV-1 specific treatment
Labial herpes simplex episodes	Pritelivir (AIC316), Topical		II	NCT02871492	
Herpes simplex infection	Monoclonal gD-antibody (UB-621), Subcutaneous	Monoclonal gD-antibody	I	NCT02346760	
			II	NCT03595995 (HSV-2)	
Prevention of recurrent herpes labialis	SQX770 (squaric acid dibutyl ester), Topical	Enhancement of immune system	II	NCT02965781	Immunomodulatory treatment
Prevention of herpes keratitis recurrences	Prednisolone acetate and prednisolone sodium phosphate (corticosteroids), Topical	Anti-inflammatory, adjuvant for oral acyclovir or valacyclovir	IV	NCT03626376	
Herpes simplex encephalitis related conditional deficiencies	Dexamethasone (corticosteroid), Intravenous	Anti-inflammatory, adjuvant for standard care	III	NCT03084783	
Herpes simplex labialis related pain	RMN3001 (Diclofenac, lidocaine), Topical	Anti-inflammatory	II	NCT02207881	

1.4.3.1 Helicase-primase inhibitors

The viral helicase-primase complex, essential for DNA replication is a novel target for herpes simplex therapy. Currently, two helicase-primase inhibitors (HPI), amenamevir (ASP2151) and pritelivir (AIC316) are studied in clinical phases III and II, respectively. Of the two, amenamevir is studied for a wider range of indications, including also genital herpes simplex infection, and is administered orally, whereas pritelivir is an ointment yet only studied for labial infections. Additionally, amenamevir has demonstrated *in vivo* efficacy against corneal HSV-1 infection (Sasaki et al., 2013), suggesting it's potential also for treatment of multi-drug resistant herpes keratitis strains in the future. Amenamevir's success in the phase III trial is not yet reported. Pritelivir, however, is reported to have had success in its phase II trials, which are currently ongoing with immunocompromised individuals.

1.4.3.2 Vaccine development

To this date, no HSV-1 nor HSV-2 vaccine is approved, however, many vaccine trials targeting especially HSV-2 are ongoing. Some vaccines have already made it far along the pipeline, such as Herpevac, which eventually was unsuccessful. The capability of HSVs to evade the adaptive immunity can be considered one of the reasons for struggles in the development of vaccines.

Herpevac is an investigative gD-vaccine, that unfortunately failed to meet its primary endpoint at phase III (NCT00057330). It was not efficient against its primary target HSV-2, yet it demonstrated efficacy against genital HSV-1 (Belshe et al., 2012). Currently, a gD-antibody (UB-621) intended for treatment of herpes simplex episodes, has successfully finished phase I against HSV infection (NCT02346760) altogether and continued to an ongoing phase II to treat genital HSV-2 infection (NCT03595995).

In contrast to subunit vaccines, such as the gD-vaccine, replication defective viruses have been studied in vaccine development. First of these vaccines in clinical trials is the HSV529-vaccine (a replication defective HSV-2 *dl*5-29). In its phase I trial (NCT01915212), it was shown safe, and effective in increasing antibody levels in seronegative and seropositive individuals (Dropulic et al., 2019). The vaccine has recently been taken to phase II trials (NCT04222985) likely with four different

immunotherapeutic modifications to the replication deficient virus. The trial is currently recruiting.

1.4.3.3 Immunomodulation

Even though immunomodulation is not exactly HSV-specific treatment, it is a major part of the current investigative clinical pipeline of HSV-treatment, and thus included here.

Corticosteroids are studied by a phase IV trial investigating two corticosteroid adjuvants to support current treatment for stromal keratitis (NCT03626376) and a phase III trial with adjuvant corticosteroid therapy to reduce cognitional deficits related to herpes encephalitis (NCT03084783). Corticosteroids have been suggested to be efficient as adjuvant treatments for herpetic diseases (Arain et al., 2015; Lizarraga et al., 2013; Wilhelmus et al., 1994). However, in general their use is controversial since they effect the natural defense system against herpes (Ramos-Estebanez et al., 2014). To this date, the keratitis trial is still recruiting, and the encephalitis trial has not begun, so no results are yet reported.

Anti-inflammatory treatment is also studied for herpes labialis (NCT02207881), yet not with corticosteroids but with a topical diclofenac-lidocaine mixture aiming to decrease labial herpes infection related pain and inflammation. No results have been yet reported of the trial.

Additionally, squaric acid dibutyl ester (SQX770), was studied in phase II (NCT02965781) as an enhancer of the immune system to prevent labial herpes recurrences. Based on phase II results, it is reported to be safe, and effective (PR newswire, 2019). As suggested by phase I results, the efficacy to prevent recurrences is based on induced interferon γ levels and decreased interleukin 5 levels (McTavish et al., 2019).

1.5 RNA interference as an antiviral approach

1.5.1 Mechanism of RNAi

RNA interference (RNAi) is a natural, conserved mechanism, which leads to RNA mediated regulation of gene expression (Cerutti and Casas-Mollano, 2006; Fire et al., 1998). There are multiple short RNA species that lead to RNAi, such as microRNAs, which are regulatory elements encoded by genomes of plants, animals and viruses, including HSV (Jurak et al., 2010), and short interfering RNAs (siRNAs), which lead to sequence specific mRNA degradation. siRNAs are widely used as tools for research, as wells as recognized for their potential in therapeutic use for example in interfering with viral replication.

The siRNAs function by a multiprotein complex; the RNA-induced silencing complex (RISC). Argonaut 2 protein is an endonuclease, and an essential component of RISC. It utilizes both strands of the siRNA to find mRNA complementary to either of the strand's sequence, eventually leading to cleavage and degradation of the found mRNA (Rand et al., 2005). The strand complementary to the mRNA is referred as the antisense or the guide strand, whereas the other strand is referred as the sense or the passenger strand. It has been shown that exogenous dsRNA is most potent in RNAi at the length of approximately 27 bp (Kim et al., 2005). dsRNAs of such length (>21 bp) are cleaved by endogenous Dicers before entering RISC, which leads to elevated silencing potency (Kim et al., 2005).

Innate response and consequent toxicity peak related to dsRNA length is at 88 bp (Jiang et al., 2011). However, generally, the longer the exogenous dsRNA, the higher the cellular innate response. Importantly, at dsRNA lengths from 19 to 30 bp, which is the common length of siRNAs, no significant elevation in toxicity or interferon response is detected (Reynolds et al., 2006).

1.5.2 Antiviral siRNA therapy

siRNAs are currently recognized as having therapeutic potential against emerging pathogens and otherwise untreatable pathogens, including those that have treatments with only limited effectiveness, and those that are in risk of developing drug resistance (Levanova and Poranen, 2018). The antiviral siRNA approach is in

general extremely straight-forward, and for initiation of therapy development, basically only a genomic sequence is required. Importantly, genomic data is nowadays available almost immediately after discovery of a novel pathogen (Chan et al., 2020). The *in silico* designed antiviral siRNAs can then be generated either chemically or enzymatically for *in vitro* assessment of safety and efficacy (Levanova and Poranen, 2018).

First siRNA to receive market approval was an intravenous siRNA under the tradename Onpattro™ for treatment of polyneuropathy caused by hereditary transthyretin-mediated amyloidosis, which was approved by European Medicines Agency and the U.S. Food and Drug Administration in 2018 (Hoy, 2018). The active siRNA of Onpattro™ is delivered by a lipid nanoparticle to hepatocytes. Recently, the same company received marketing authorization for another siRNA drug for treatment of acute hepatic porphyria (Scott, 2020). Currently, no other siRNAs, and thus also no antiviral siRNAs are yet on the market. Nevertheless, various antiviral siRNAs have been studied in clinical trials. These trials have studied treatment of respiratory syncytial virus (RSV), hepatitis B virus, hepatitis C virus, HIV-1 or Zaire Ebolavirus (Levanova and Poranen, 2018). Most recently also human papilloma virus targeted siRNA begun clinical trials (recruiting, NCT04278326). However, no siRNAs targeting herpesviruses have yet reached clinical trials.

siRNAs offer a valuable approach with a novel mechanism of action for treatment of HSV. The treatment would benefit not only those patients suffering from episodes caused by drug resistant strains of HSV, such as numerous herpes keratitis patients, but also those in need of a more efficient treatment, such as herpes encephalitis patients. Moreover, as the diseases caused by herpes simplex viruses are most commonly topical (eye, skin, mucosa), the delivery of siRNA is not as challenging, as with internal, systemic treatment. As topical treatments lead only to low systematic load of siRNA (DeVincenzo et al., 2008), the overall adverse effects are minimal. Furthermore, intranasal delivery of siRNA could reach the central nervous system (Rodriguez et al., 2018), and thus enhance treatment of herpes simplex encephalitis (da Silva et al., 2016), or eventually even target the latent infection of HSV.

siRNAs against HSV have not yet been studied in clinical trials but have been studied *in vitro* and *in vivo* with promising results. Of the targets studied, UL39 and UL29

targeted siRNAs have demonstrated efficacy against various patient derived strains *in vitro* (Paavilainen et al., 2016; Silva et al., 2014), as well as efficacy *in vivo* (da Silva et al., 2016; Paavilainen et al., 2017), encouraging for further research with these targets. Moreover, as UL29 has high homology between HSV-1 and HSV-2, siRNAs targeting UL29 have demonstrated *in vivo* efficacy against both of the viruses (Paavilainen et al., 2017; Palliser et al., 2006). Altogether in *in vivo* studies (please see **Table 4**), siRNAs against HSVs have led to decreased symptoms and mortality, as well as to significant inhibition of virus production inhibition in the infected tissues (da Silva et al., 2016; Li et al., 2014; Paavilainen et al., 2017; Palliser et al., 2006), thus supporting the potential of siRNA therapy against herpes simplex viruses.

Table 4 – *In vivo* treatment of HSV with siRNA

Target (gene|protein)		Model	siRNA delivery method	Reference
UL29, UL27	ICP8, gB	*In vivo* intravaginal infection	Intravaginal delivery, lipid-based	(Palliser et al., 2006)
α4	ICP4	*In vivo* corneal infection	Topical application to cornea, siRNA-polymer complex	(Li et al., 2014)
UL29	ICP8	*In vivo* corneal infection	Topical application to cornea, no delivery reagent	(Paavilainen et al., 2017)
UL39	ICP6	*In vivo* CNS infection	Intracaudal inoculation, siRNA-glycoprotein complex	(da Silva et al., 2016)

1.5.3 Challenges in antiviral siRNA therapy

siRNA therapy faces challenges in their stability, delivery, off-target effects as well as limited target size and related target optimizations. Viruses as a target for siRNA therapy increase the importance of choosing a relevant target sequence and highlight the need for the siRNA therapy to tolerate target variation, since variability within and between virus strains exist. Additionally, some RNA-viruses can suppress the RNA interference pathway (Fabozzi et al., 2011; Li et al., 2016), however, such mechanism is not (yet) described with herpesviruses. Anyhow, due to the high potential of the siRNAs altogether, solutions to these previously mentioned challenges are and have been widely studied.

The delivery challenges are related to the negative, anionic charge of RNA. Due to the anionic charge, they cannot passively diffuse trough the cellular membrane. Additionally, siRNAs are rapidly degraded by nucleases if not protected (DeVincenzo et al., 2008). A common method to overcome the low stability are chemical modifications. Popular modifications are those at the 2'-position in the ribose, which are proven not to interfere with RNAi, but even increase siRNA potency and stability both *in vitro* (Allerson et al., 2005) and *in vivo* (Manoharan et al., 2011). The delivery challenges of siRNAs may be solved in many ways, for example by targeted viral vectors, nanoparticles or conjugates, such as lipids or antibodies (Levanova and Poranen, 2018). Moreover, delivery reagents suitable for both *in vivo* and human use (Sidi et al., 2008), which have also enabled siRNA mediated antiviral efficacy *in vivo* (Rodriguez et al., 2018), are nowadays commercially available. Additionally, siRNAs have proven antiviral efficacy even without a delivery reagent *in vivo* upon topical, corneal administration against HSV-1 (Paavilainen et al., 2017) and in humans via intranasal administration of siRNA aerosol targeted against RSV (DeVincenzo et al., 2010).

The off-target effects of siRNAs can be caused by various reasons addressed by strategies in careful siRNA design (Gatta et al., 2018). Primarily, the off-target effects are mediated by similar sequences of nontargeted mRNAs leading to nonspecific gene silencing. They can also be mediated by matches between the seed region (nucleotides 2-7 or 2-8 in the antisense strand) and untranslated mRNA regions (Birmingham et al., 2006). In addition, off-target effects include elevated interferon responses induced by certain sequence motifs, which can lead to cytotoxicity (Fedorov et al., 2006; Meng and Lu, 2017) These toxic effects can be reduced by chemical modifications at the 2'-position of the ribose (Fedorov et al., 2006). The chemical 2'-modifications have also shown to reduce the off-target effects related to nonspecific gene silencing (Jackson et al., 2006).

The last challenge mentioned is the limited target size of siRNA, which manifests as a low tolerability of mutations in the viral genome and as high odds to surfacing of escape mutants. Also, predicting actions of single, individual siRNAs is not possible, since the *in silico* analysis do not take secondary structures of target transcripts nor possible surrounding protein complexes into account (Parsons et al., 2009). In general, mutation tolerability of siRNA depends on site of the mutations with regard

to the siRNA (Amarzguioui et al., 2003). In the central region of the siRNA, which is generally the most sensitive for mutations, a double mutation has been shown to lead to total loss of activity (Holen et al., 2002). Independent of to the position, decrease in silencing ability is detected already at one and two nucleotide mismatches (Dahlgren et al., 2008; Holen et al., 2002). The issues of low mutation tolerability can be partially solved with pooling multiple siRNAs (Knoepfel et al., 2012; O'Brien, 2007). The pooling increases possibilities for successful, sequence specific target knock-down (Parsons et al., 2009), but nevertheless requires thorough *in vitro* and *in silico* evaluation of the individual siRNAs within the pool. Regarding antiviral siRNAs, the pools enable overcoming possible variation in target pathogen transcripts. Simultaneously, by targeting multiple sequences, the sequence-dependent off-target effects dilute (Hannus et al., 2014). siRNA pool may be produced synthetically, but faster and more straightforward is their enzymatical synthesis (Levanova and Poranen, 2018). A recently developed method enables a straight forward enzymatical production of siRNAs from long target sequences reaching up to 3.5 kbp (Nygårdas et al., 2009; Romanovskaya et al., 2012). The siRNA pool created in such a way is referred to as an siRNA swarm, since a siRNA swarm is not exactly a pool of few individual siRNAs, but a swarm of siRNAs targeting a long RNA sequence back-to-back. siRNA swarms are more thoroughly introduced in the next chapter.

1.5.4 siRNA swarms

siRNA swarms represent a collection of 27 bp (25 bp and 2 nt overhangs) long siRNAs, which are enzymatically produced from a long dsRNA representing the pathogen target sequence (Romanovskaya et al., 2012). Depending on the application, the target sequence may be coherent, or a chimeric combination of multiple genes (Jiang et al., 2019). Enabling of longer targets decreases the possibility of cumulative off-target effects as well as the significance of genetic diversity and point mutations, which are often a great concern especially in long-term infections and their treatment. Thus, siRNA swarms answer the challenges of traditional antiviral siRNAs, such as their susceptibility to off-target effects and to the genetic diversity of their targeted pathogen populations.

siRNA swarms have proven efficient against different viral pathogens (Jiang et al., 2019; Nygårdas et al., 2009; Romanovskaya et al., 2012) and are well tolerated by various cell types (Jiang et al., 2019; Paavilainen et al., 2015). They have already shown comparable efficacy against multiple circulating strains and reference strains, regardless of their genetic deviation. This phenomenon is demonstrated not only for HSV strains (Paavilainen et al., 2016), but also for influenza virus A (Jiang et al., 2019) and enteroviruses, where one siRNA swarm was able to significantly inhibit not only different strains, but different enteroviruses (Nygårdas et al., 2009).

The most extensively studied siRNA swarm target is HSV-1 and its UL29 gene. The UL29 targeting siRNA swarm has been shown least immunostimulatory and the most antiviral of the HSV-1 specific siRNA swarms tested (Paavilainen et al., 2016). Moreover, the UL29 siRNA swarm does not cause unfavorable elevated interferon responses in retinal, epithelial nor neuronal cells (Paavilainen et al., 2016; Paavilainen et al., 2015). In addition to the high efficacy against various patient derived strains *in vitro* (Paavilainen et al., 2016), treatment with UL29 siRNA swarm decreased fatality and improved the clinical symptoms of a corneal infection *in vivo* (Paavilainen et al., 2017). Currently, the UL29 siRNA swarm is the most promising siRNA candidate for treatment of HSV-1.

1.6 Summary

The multiple severe diseases caused by HSV-1, the lack of a cure or vaccine, and the emerged resistance to the current treatment-of-choice facilitate a need for a drug against HSV-1 with a novel mechanism of action and tolerability for sequence variability between and within HSV-1 strains. The siRNA swarms are a potential drug candidate in development to answer this need. Yet, siRNAs are challenged by their fast degradation *in vivo*. This challenge can be overcome by 2'-modifications in the nucleotides of siRNA. One of the modifications, which is shown to improve *in vivo* stability of siRNAs, are 2'-fluoro-modifications (Manoharan et al., 2011). Thus, the UL29 siRNA swarm was incorporated with 2'-fluoro-modifications. In this Master's thesis, altogether six different modified siRNA swarms are compared. In short, the goal of the studies is to elucidate whether chemically modifying the siRNA swarms against HSV-1 is beneficial for the therapeutic or prophylactic antiviral activity, or their duration, and whether the modifications significantly change the host response profile to siRNA swarms in relevant target tissues of HSV-1. The experiment flow is presented **Figure 3**.

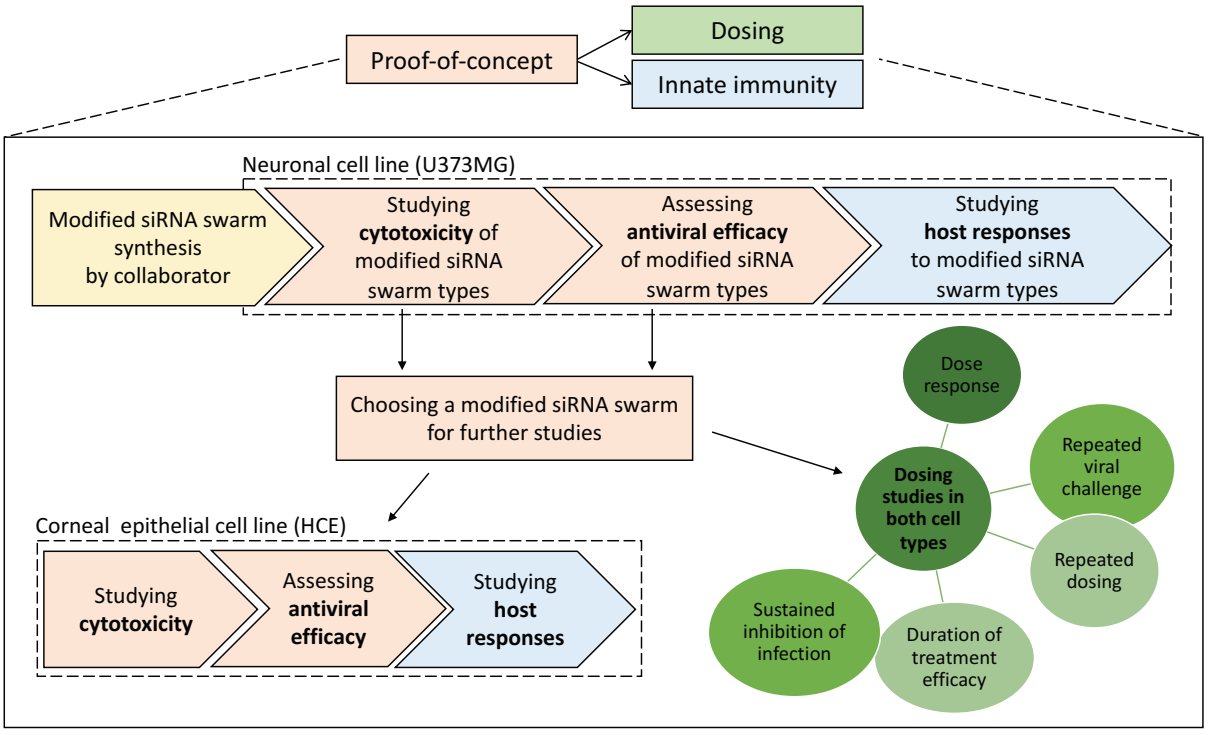

Figure 3 - Flow of the experiments

2 Results

2.1 Proof-of-concept studies

The 2′-fluoro-modified siRNA swarms, synthetized by our collaborator at the University of Helsinki, represented modifications of adenosine (A), cytidine (C) or uridine (U) in the antisense strand of the siRNA sequence. The strand was either partially (10%) or fully (100%) modified regarding the nucleotide studied. All the antiviral siRNA swarms used targeted the UL29 an essential gene of HSV-1. For a list of the siRNAs used, please see materials and methods (*4.1 siRNA swarms and control RNAs used)*. The cell lines used throughout the studies are HCE and U373MG. For clarification of cell lines used, please see materials and methods (*4.2 Cell lines used*).

2.1.1 Cellular viability after transfection with modified siRNA swarms

First, the cytotoxicity of the modified siRNA swarms was assessed in both cell lines. The positive control for cytotoxicity, 88 bp dsRNA, caused a significant drop in cellular viability ($p \leq 0.001$) of 80% and 60% in U373MG and HCE cells, respectively (**Figure 4A** & **Figure 4B**). In both cell lines, all the modified siRNA swarms were tolerated at the same approximate level compared to the water transfected cells. Transfection with water (mock treatment), representing lipofectamine RNAiMAX without any RNA resulted in slight decrease of cellular viability (appr. 15%–25%) compared to untreated cells. In comparison to the nonmodified siRNA swarms, the different types of modified swarms were equally tolerated in U373MG cells. However, in the HCE cells the fully and partially cytidine modified (100% F-C and 10% F-C, respectively) as well as the fully uridine modified (100% F-U) siRNA swarms led to significant decrease in cellular viability (for 100% F-U and 10% F-C $p \leq 0.05$, and for 100% F-C $p \leq 0.01$).

As a follow-up study, a wider dose range (12.5 nM – 150 nM) was studied with 10% and 100% uridine modifications (**Figure 4C** & **Figure 4D**). Even the tripling of the concentration had no effect on the tolerability, and the modified siRNA swarms did not differentiate from the nonmodified siRNA swarm profile in this sense.

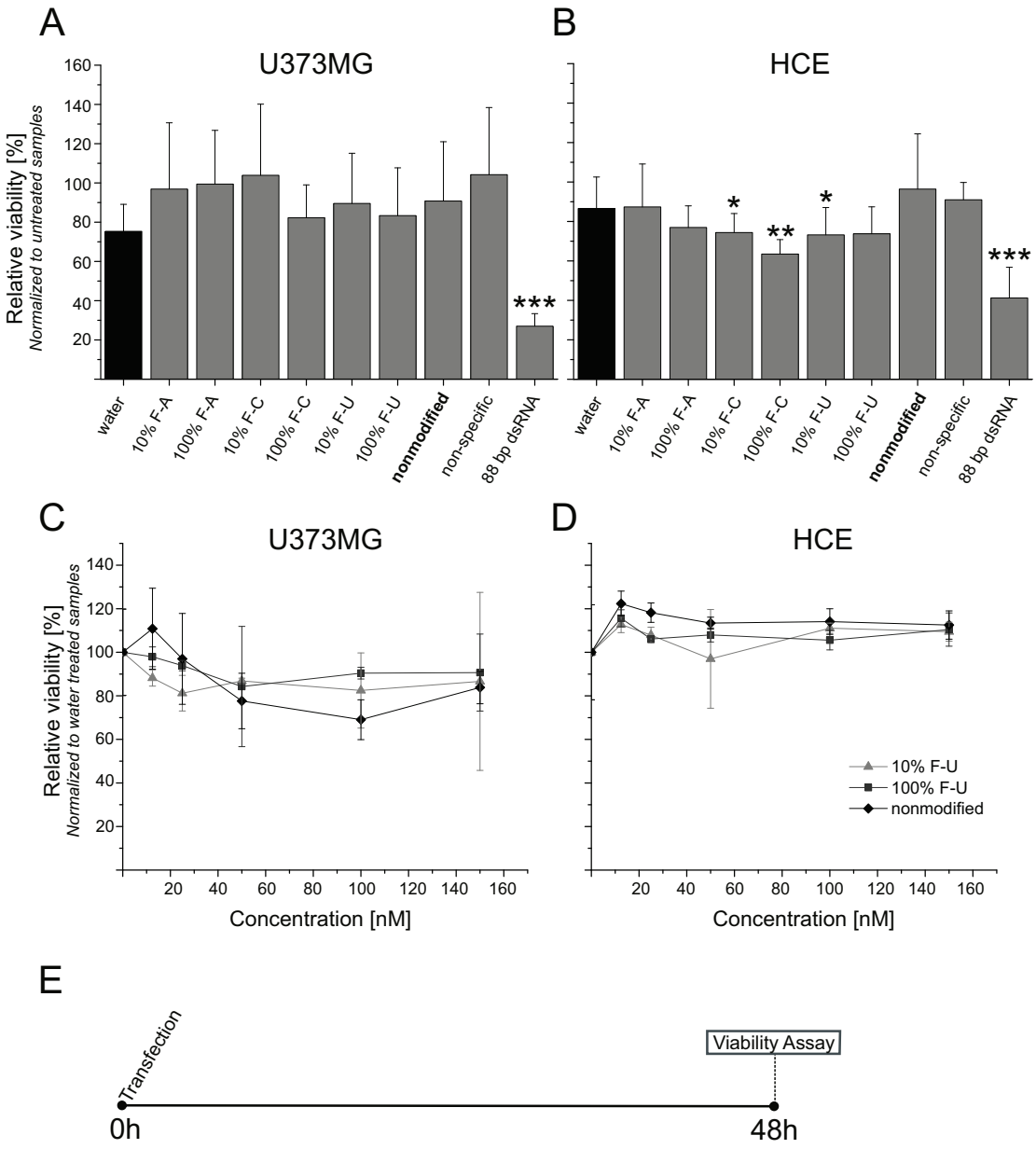

Figure 4 – Effects of the modified siRNA swarms on cellular viability. Human glioma (U373MG) (A, C) and human corneal epithelial (HCE) (B, D) cells were transfected at the RNA concentration of 100, 50, 25, or 12.5 nM of the indicated siRNA swarms or 12.5 nM of the cytotoxic control (88 bp dsRNA), left untreated or transfected with transfection reagent only (mock; water transfection). At 48 hours post transfection (hpt) the cellular viability was quantified with a luminescent assay. **(A, B)** The relative viability of the treated cells, at the RNA concentration of 50 nM is presented as the percentage of viability of the untreated cells. The bars represent the mean and the whiskers the standard deviation of the mean (N≥8 per treatment, data from two individual experiments). The p-values are calculated against the relative cytotoxicity of the nonmodified siRNA swarm (*, p≤0.05; **, p≤0.01; ***, p≤0.001). **(C, D)** The relative cytotoxicity of the indicated siRNA swarms is shown relative to the mock treated control. The whiskers represent the standard deviation of the mean (N≥3 per treatment). **(E)** Experiment flow. The cells were transfected, and 48 hours later measured for viability.

2.1.2 Prophylactic antiviral assay with modified siRNA swarms

The antiviral assays were conducted utilizing a previously used model of prophylactic treatment, where the siRNA swarms are transfected into the cells four hours before infection (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012). The virus amount was quantified by plaque titration of the virus (titration) from culture supernatant and by measuring viral mRNA levels by quantitative real-time PCR (RT-qPCR) (**Figure 5**). The samples for RT-qPCR were derived from cellular lysates, from which RNA was extracted and converted into complementary DNA (cDNA) by reverse transcriptase (RT) reaction. The viral titration result derived from the culture supernatant reflects the virus amount shed from the infected cells as a result of viral replication. The amount of viral shedding is presented as plaque forming units (pfu) per milliliter. Please see materials and methods (*4.7 Plaque titration of the virus*) for clarification of viral plaque titration. The RT-qPCR measured the mRNA expression of a viral replication protein of HSV-1 [viral protein 16 (VP16) also known as α Trans-Inducing Factor (α-TIF)], which was normalized to expression of a housekeeping gene (Glyceraldehyde 3-phosphate dehydrogenase, abbr. GAPDH). As for U373MG, all types of modified siRNA swarms were analyzed, but for HCE cells, only adenosine modified siRNA swarms were used. The adenosine modified siRNA swarms were chosen for use with HCE cells, since upon confirmation of antiviral efficacy, minimal interference of toxic cellular responses was preferred (**Figure 4B**).

The titration results (**Figure 5A** & **Figure 5B**) confirmed that the HSV-specific siRNA swarms, both modified and nonmodified decreased the amount of viral shedding by approximately four orders of magnitude in U373MG cells (>99.9% inhibition) and well over one order of magnitude in HCE cells (>90% inhibition) compared to the nonspecific siRNA swarm control at the siRNA swarm concentration of 50 nM. The difference of titer compared to the nonspecific siRNA swarm treatment was statistically significant ($p \leq 0.001$) for each of the antiviral treatment groups in both cell lines. Additionally, in U373MG cells, the fully cytidine modified siRNA swarm, 100% F-C, revealed significantly increased antiviral efficacy compared to the HSV-specific nonmodified siRNA swarm. On the contrary, the other fully pyrimidine modified swarm, 100% F-U, was significantly less antiviral than the nonmodified HSV-specific

siRNA swarm. These significant differences between the nonmodified and the modified antiviral siRNA swarms were not demonstrated in the mRNA analysis of VP16 (**Figure 5C** & **Figure 5D**). However, the VP16 mRNA level comparisons complemented the titration results regarding the significant decrease in viral mRNA levels compared to the nonspecific treatment in both cell lines and all types of HSV-specific siRNA swarms studied ($p \leq 0.05$ in HCE, $p \leq 0.001$ in U373MG).

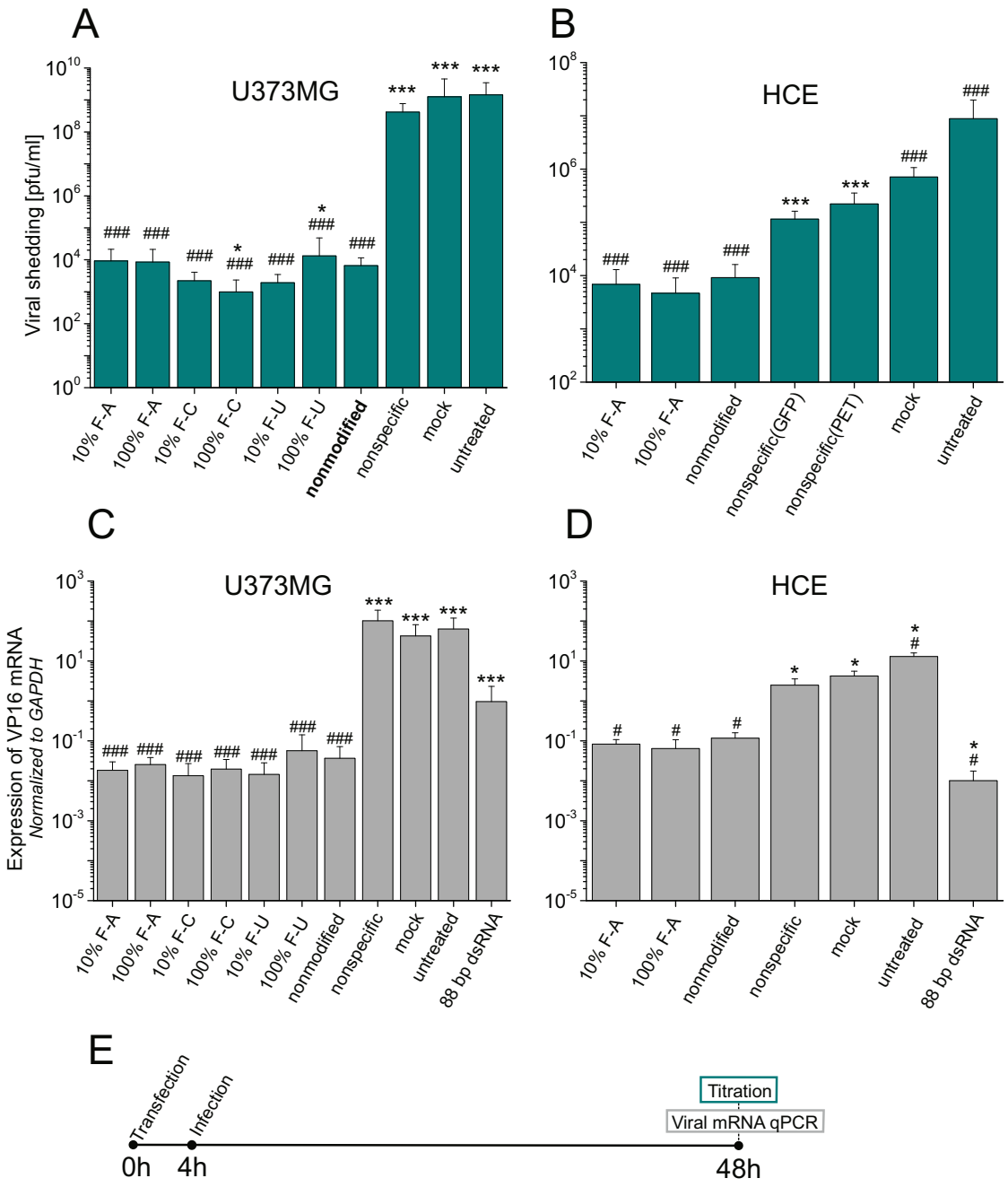

Figure 5 – Prophylactic antiviral efficacy of modified siRNA swarms. Human glioma (U373MG) and human corneal epithelial (HCE) cells were transfected with

50nM of modified anti-HSV (anti-UL29) siRNA swarms, nonmodified anti-HSV (anti-UL29) swarm, nonspecific control swarm or 10nM of 88 bp dsRNA (positive toxicity control). The modified siRNA swarms had all (100%) or a part (10%) of the A, C or U in their antisense strand 2'-fluoro-modified. Here, in U373MG experiments the nonspecific control swarm targeted a bacterial lac-operon sequence (PET), and in HCE experiments a GFP-sequence, unless otherwise stated. Additional non-RNA controls used were the untreated samples and water treated samples (mock), which represents the effect of transfection reagent alone. Four hours post transfection (hpt) the cells were infected with 1000 pfu per well of HSV1-GFP. **(A, B)** Viral shedding, which represents the virus replicated and exited from the cells to the supernatant, was quantified at 48 hpt from U373MG (A) and from HCE (B) culture supernatant. The unit, plaque forming units (pfu), describes the quantity of viral plaques resulting from infection with one milliliter of the collected sample. **(C, D)** VP16 mRNA expression was quantified at 48 hpt from U373MG (C) and from HCE (D) cells via RT-qPCR and normalized to housekeeping gene (GAPDH) expression. In panels A, B and C, the data is from two different experiments (N per repeat ≥ 4), except for PET treated cells in panel B, which represent one subexperiment only. In panel D, the data is from one experiment (N per treatment ≥ 4). The columns represent the mean+SD of the indicated treatment groups. The p-values are presented against the nonmodified siRNA swarm treated samples (*, p≤0.05; *** p≤0.001) and against the nonspecific siRNA swarm treated samples (#, p≤0.05; ##, p≤0.01; ### p≤0.001). If not shown, the p-value was nonsignificant. **(E)** Experiment flow. The cells were transfected four hours before infection (prophylactic model), and 48 hours later the virus production was quantified using two methods: titration and RT-qPCR measuring VP16 mRNA expression.

Interestingly, the nonspecific swarm targeting green fluorescent protein (GFP) had no significant difference in viral titer compared to that of the nonspecific swarm targeting a bacterial lac-operon sequence (PET), even though the virus used (HSV1-GFP) expresses GFP (please see *4.3 Viruses used*). The average titer of the cells treated with the GFP specific siRNA swarm was, however, lower than that of the cells treated with the PET siRNA swarm, but the difference was nonsignificant.

2.1.3 Host responses to modified siRNA swarms

To support viability data and confirm sequence specificity of the modified siRNA swarms, a panel of innate host responses in treated and control-treated cells were measured at the timepoint of 48 hpt. Transcripts (mRNA) of interferon beta (IFN-β) and interferon stimulated gene 54 (ISG54) as well as human myxovirus resistance proteins 1 and 2 (MxA and MxB, respectively) were quantified by RT-qPCR (**Figure 6**). All the data was normalized to GAPDH mRNA expression.

In U373MG cells, host responses to treatment with UL29 targeting modified siRNA swarms, 100% F-C, F-A, and F-U along with 10% F-C, F-A, and F-U were studied. To control the experiment, responses to a nonmodified UL29 siRNA swarm were studied, as well as to a nonspecific siRNA swarm (PET), to a cytotoxic control (88 bp dsRNA) and to transfection reagent alone (water transfection; mock). The baseline expression, shown to be positive for each of the innate immunity markers was quantified from cells left untreated. The untreated cells had significantly lower expression of MxA, MxB and ISG54 compared to the nonmodified siRNA swarm ($p \leq 0.001$). IFN-β, then again, was expressed at the same approximate level in untreated cells as with the different siRNA swarm treatments and hence had no significant difference from baseline to treated cells. The host innate responses to nonmodified siRNA swarm did not differ from those caused by the transfection reagent alone in any of the studied responses (**Figure 6;** A/C/E/G). The only modified siRNA swarm, that differed significantly from the nonmodified siRNA swarm in any of the studied responses, was 100% F-C, which had significantly elevated IFN-β ($p \leq 0.01$) and ISG54 ($p \leq 0.05$) levels compared to the nonmodified siRNA swarm. The MxA and MxB levels were very similar in each of the treatments. Even treatment with the positive cytotoxic control, 88 bp dsRNA led only to slight nonsignificant elevation of MxA and MxB levels, whereas for ISG54 and IFN-β, treatment with 88 bp dsRNA induced significantly higher response levels compared to the nonmodified siRNA control ($p \leq 0.001$).

In HCE cells, responses to treatment with modified UL29 siRNA swarms (100% F-A and 10% F-A) and the nonmodified UL29 siRNA swarm were studied, as well as to a nonspecific siRNA swarm (GFP), to a cytotoxic control (88 bp dsRNA) and to transfection reagent alone (water transfection; mock) (**Figure 6**; B/D/F/H). The baseline expression of the cells was positive with all the studied markers, but significantly lower ($p \leq 0.05$) than those of the nonmodified siRNA swarms in all studied mRNA expression levels. In contrast, the cytotoxic 88 bp dsRNA induced significantly higher responses of MxA, IFN-β and ISG54 ($p \leq 0.05$) than those of the nonmodified siRNA swarm. Overall, in HCE cells the adenosine modified siRNA swarms did not differ from the nonmodified siRNA swarm in any of the markers studied, and neither did the response to transfection reagent alone.

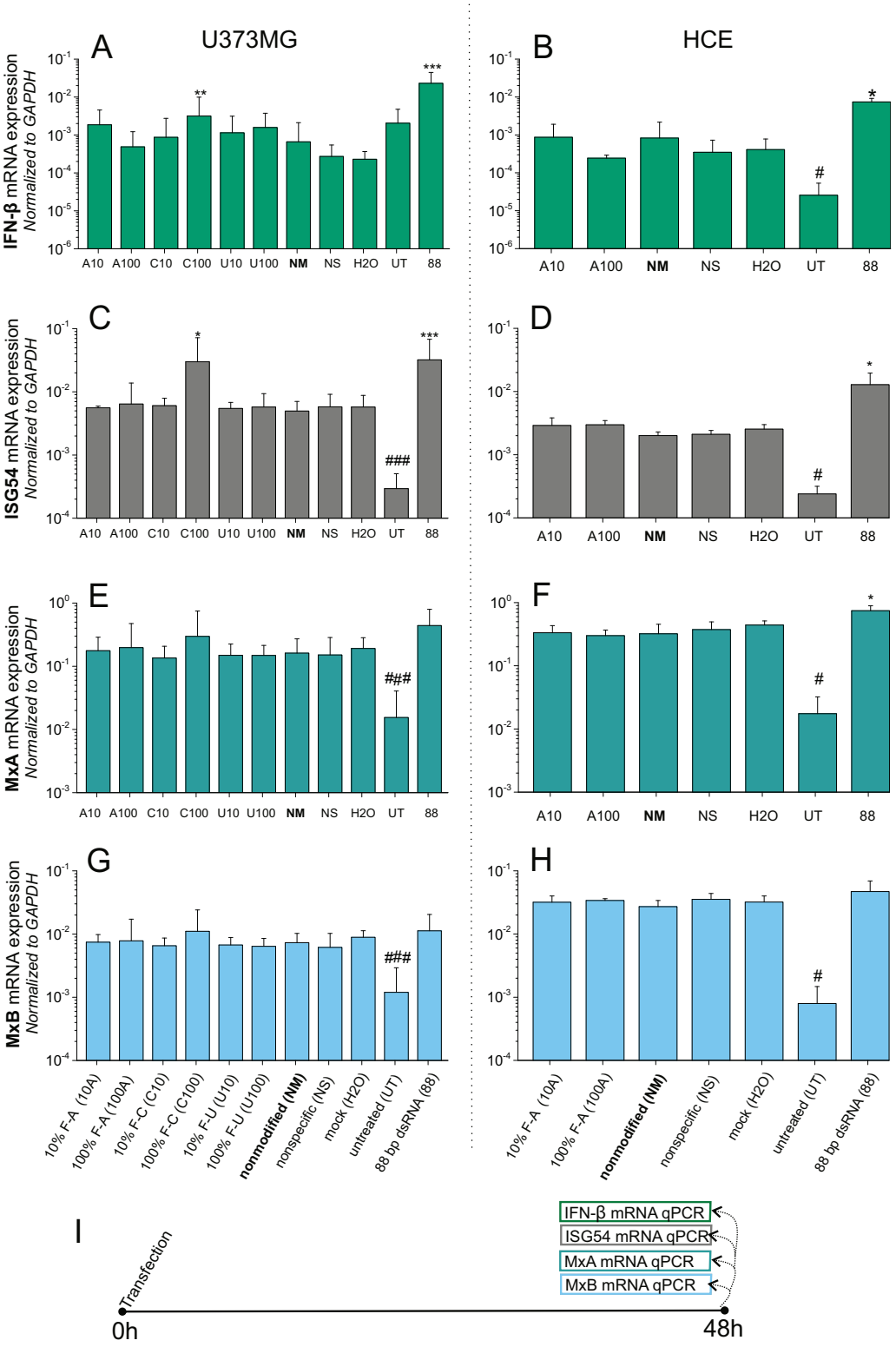

Figure 6 - Host responses to modified siRNA swarms. U373MG cells were transfected with 50nM of each of the modified siRNA swarms, containing partially or fully (10% or 100%, respectively) modified nucleotides (A, C or U). Nonmodified and nonspecific control siRNA swarms (50 nM), as well as cytotoxic control (88 bp dsRNA) (10 nM) in addition to water transfected (mock) and untreated controls were used. HCE cells were treated similarly, but only adenosine modified siRNA swarms

(100% F-A and 10% F-A) were studied. The nonspecific swarm used targeted GFP in studies with HCE cells and bacterial lac operon in U373MG studies. The treatments and their abbreviations are listed in the lower panel legends. **(A, B)** Interferon beta (IFN-β), **(C, D)** interferon stimulated gene 54 (ISG54) **(E, F)** human myxovirus resistance protein 1 (MxA) and **(G, H)** human myxovirus resistance protein 2 (MxB) mRNA expressions were quantified at 48 hpt with RT-qPCR and normalized against GAPDH mRNA expression. The columns represent the mean+SD of two individual experiments (U373MG) (N≥8 per treatment group) or one experiment (HCE) (N≥4 per treatment group). The significant increases (*, p≤0.05; **, p≤0.01; ***, p≤0.001), and the significant decreases (#, p≤0.05; ##, p≤0.01; ### p≤0.001) compared to the nonmodified siRNA swarm are shown. If no p-value is presented, the difference was not significant. **(E)** Experiment flow. The cells were transfected and 48 hours later quantified for mRNA of relevant innate immunity markers via RT-qPCR.

2.2 Dosing studies

According to the proof-of-concept studies, relevant modified siRNA swarms were chosen for the dosing studies. For the dose-response study (chapter *2.2.1*), adenosine modified siRNA swarms were chosen, since they demonstrated minimal immune responses and cytotoxicity in both cell types in contrast to other modification types (**Figure 4, Figure 5**). Thus, if elevated antiviral potency or efficacy in comparison to the nonmodified siRNA swarm were to be detected, it would be due to presence of modifications in the siRNA swarms with no interference from cellular antiviral responses induced by 2'-fluoro-nucleotides or 2'-fluoro-DsiRNA. In the other dosing studies (chapters 2.2.2, 2.2.3, 2.2.4, and 2.2.5) the cytidine modified siRNA swarms were used, since in the UL29 siRNA target, the most abundant nucleotide is cytidine. The maximum number of modified nucleotides was preferred regardless of slight immune response elevation (**Figure 5**), since the experiments are related to the hypothesized prolonged stability and efficacy due to incorporation of modified nucleotides and these qualities are most likely present when the highest number of modifications are studied.

2.2.1 Dose-response relationship between modified siRNA swarms and inhibition of viral shedding

The first of the dosing studies conducted was a dose-response study, examining the relationship between the concentration of siRNA swarm and the resulting viral shedding levels of HSV-1 from the cells. For the dose response study, a prophylactic

and a therapeutic model were used. In contrast to the prophylactic model, in the therapeutic model the cells were infected before treatment to better represent a clinical situation. In both models, the infection and treatment were four hours apart. The dose range used varied from 0.41 nM to 100 nM (three-fold dilution series). The siRNA swarms used for the study were modified 10% F-A and 100% F-A, in addition to a nonmodified siRNA swarm and nonspecific siRNA swarm.

The antiviral efficacy was plotted as percent inhibition of viral shedding (%), which is calculated by comparing viral shedding from treated cells to that of the untreated cells. Based on the created data, sigmoidal dose-response curves were fitted. From the curves, maximum efficacy (E_{max}), its standard error (SE) and the concentration resulting in half of maximal efficacy (EC_{50}) were determined for all HSV-specific treatments. The results are presented in **Table 5** (U373MG) and **Table 6** (HCE).

Table 5 - Resulted dose-response parameters from U373MG cells

	Prophylactic model			*Therapeutic model*		
	10% F-A	**100% F-A**	**nonmodified**	**10% F-A**	**100% F-A**	**nonmodified**
E_{max}	100%	100%	100%	100%	100%	100%
SE	0.00013	0.00003	0.00019	0.00005	0.00002	0.00010
EC_{50}	*Not determined*					

Table 6 - Resulted dose-response parameters from HCE cells

	Prophylactic model			*Therapeutic model*		
	10% F-A	**100% F-A**	**nonmodified**	**10% F-A**	**100% F-A**	**nonmodified**
E_{max}	100%	99%	98%	99%	95%	91%
SE	0.04	0.29	0.10	0.05	0.38	0.72
EC_{50}	2 nM	4 nM	6 nM	2 nM	2 nM	3 nM

In U373MG cells, the lowest tested dilution resulted still in over 80% inhibition of virus production with all the HSV-specific siRNA swarms in both models. In the therapeutic model, the lowest concentrations were not as antivirally efficient as their

counterparts in the prophylactic setting. Nevertheless, in both models the treatment with HSV-specific siRNA swarm, whether fully, partly or not modified, resulted in a maximum efficacy of 100% (**Table 5**). However, the dose-response curves shapes suggest higher potency of the modified siRNA swarms compared to those of nonmodified siRNA swarms (not shown) in both models. Especially at the lowest concentrations the treatment with modified siRNA swarms resulted in higher antiviral efficacy than treatment with nonmodified siRNA swarms. In general, the treatment with the nonspecific siRNA swarm differed significantly ($p \leq 0.05$) from each of the specific treatments at each concentration used (data not shown).

In HCE cells, the differences between the models and treatments were more evident. The prophylactic model resulted in slightly higher (1-7%) maximum efficacy values than those of the therapeutic model with each of the treatments (**Table 6**). On the contrary, the EC_{50} values were 2-3 nM lower in the therapeutic model compared to the prophylactic model with fully modified (100% F-A) and the nonmodified siRNA swarms. For the partly modified siRNA swarm (10% F-A), the EC_{50}-value was equal between the models (2 nM). Overall, the modified siRNA swarms demonstrated higher maximum antiviral efficacy and higher antiviral potency than the nonmodified siRNA swarms in both of the models (**Table 6**). In the prophylactic model, all the specific treatments resulted in significantly lower viral titers compared to the nonspecific control (and thus higher antiviral efficacy) at concentrations higher than 1nM (data not shown). In the therapeutic model, the significances did not have such a clear pattern. However, the data indicates that the specific siRNA swarm treatment begins to differ from the nonspecific siRNA swarm treatment at a concentration of 1.23 nM in both models in HCE cells (data not shown).

2.2.2 Cellular stability of antivirally active siRNA swarms

The study was conducted with the prophylactic setting with alternating infection timepoints (**Figure 7**). The siRNA swarms used were 10% F-A, 10% F-C, 100% F-C, nonmodified and nonspecific (GFP). 10% F-A was included in addition to the cytidine modified to provide information on how a minimal number of modifications would affect the stability of active siRNA. Additionally, water transfection (mock), untreated cells and a short, single site siRNA (UL29.2) (Palliser et al., 2006) targeting

a part of the same sequence as the antiviral siRNA swarms, were used as a control. At 48 hours post infection, the supernatants were collected and quantified for viral shedding by plaque assay. For the experiment design, please see **Figure 7C.**

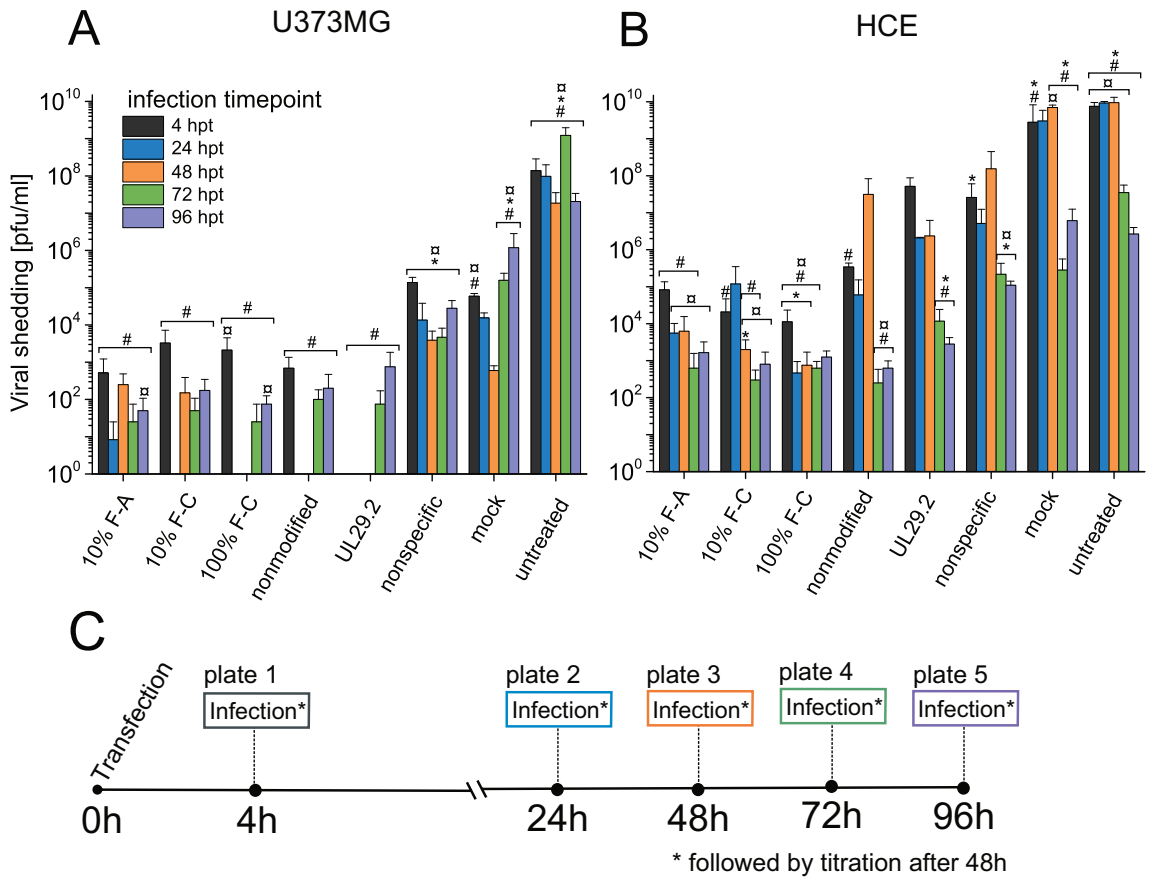

Figure 7 – Cellular stability of antivirally efficient transfected siRNA. To assess the duration of the antiviral response resulting from the transfected siRNA swarms, U373MG **(A)** and HCE **(B)** cells were transfected with 50nM of the indicated modified siRNA swarms (10% F-A, 10% F-C and 100% F-C), a nonmodified control siRNA swarm or a single site siRNA (UL29.2) all targeting the same UL29 gene of HSV-1. Nonspecific siRNA swarm (GFP) was used as a control at 50 nM in addition to untreated cells and water transfected (mock) cells (for transfection reagent effect). The cells were infected with 1000 pfu of HSV1-GFP at 4, 24, 48, 72, or 96 hours post transfection. At 48 hours post infection the supernatants of the cell cultures were collected and titrated for quantitation of viral shedding. The columns and whiskers represent the mean+SD from ≥4 biological replicates. The p-values were calculated against the nonspecific control (#, p≤0.05), nonmodified control (*, p≤0.05) and the single-site siRNA control (¤, p≤0.05) **(C)** Experiment flow. The cells were transfected followed by infection after 4, 24, 48, 72, or 96 hours. The viral shedding was quantified by titration 48 hours post infection.

In U373MG cells, all treatments targeting UL29, modified and nonmodified siRNA swarms, as well as single site siRNA were active at all timepoints studied with statistically significant antiviral efficacy ($p \leq 0.05$) in comparison to the nonspecific treatment (**Figure 7A**). None of the modified siRNA swarms differed in antiviral response compared to the nonmodified siRNA swarms at any of the timepoints studied. However, whereas the nonmodified siRNA swarm did not differ significantly from the single site siRNA, both 10% F-A and 100% F-C were significantly more antiviral than the single site siRNA at the latest timepoint. When U373MG cells were infected 24 hours post transfection, all the specific treatments except 10% F-A resulted in perfect inhibition of infection with no detectable viral shedding in the supernatant.

Complementing the results from the U373MG cells, the modified antiviral siRNA swarms did not have statistically different antiviral efficacy in HCE cells at the later timepoints compared to the nonmodified antiviral siRNA swarm (**Figure 7B**). However, they demonstrated increased antiviral efficacy ($p \leq 0.05$) compared to the nonmodified at the earlier timepoints (infection at 4-48 hpt). In contrast to the U373MG cells, in HCE cells the single site UL29.2 was significantly less antiviral ($p \leq 0.05$) than the modified siRNA swarms at the earlier timepoints (infection 4, 24 or 48 hpt) and less antiviral than the nonmodified antiviral siRNA swarm at the later timepoints (infection 72 or 96 hpt). Overall, the HSV-targeted siRNA swarms resulted to multifold lower viral shedding compared to the nonspecific siRNA swarm and 99.9% inhibition of viral shedding compared to the untreated cells even at the latest timepoints.

2.2.3 Sustained inhibition of infection by siRNA swarms

In order to study how long the infection remains inhibited in cells after transfection with 50nM of antiviral siRNA swarms, the cells were infected and then transfected (4 hpi) according to the therapeutic model. Supernatants were collected at 48, 96 and 120 hpt (2, 4, and 5 days post infection, respectively) for quantitation of reproducing virus and titrated for quantification of viral shedding (**Figure 8**). The siRNA swarms used were HSV-specific modified 10% F-C and 100% F-C, as well as their nonmodified counterpart, in addition to nonspecific (targeted to bacterial *lac*-

operon), untreated and mock-transfected controls. For the experiment design, see **Figure 8C**.

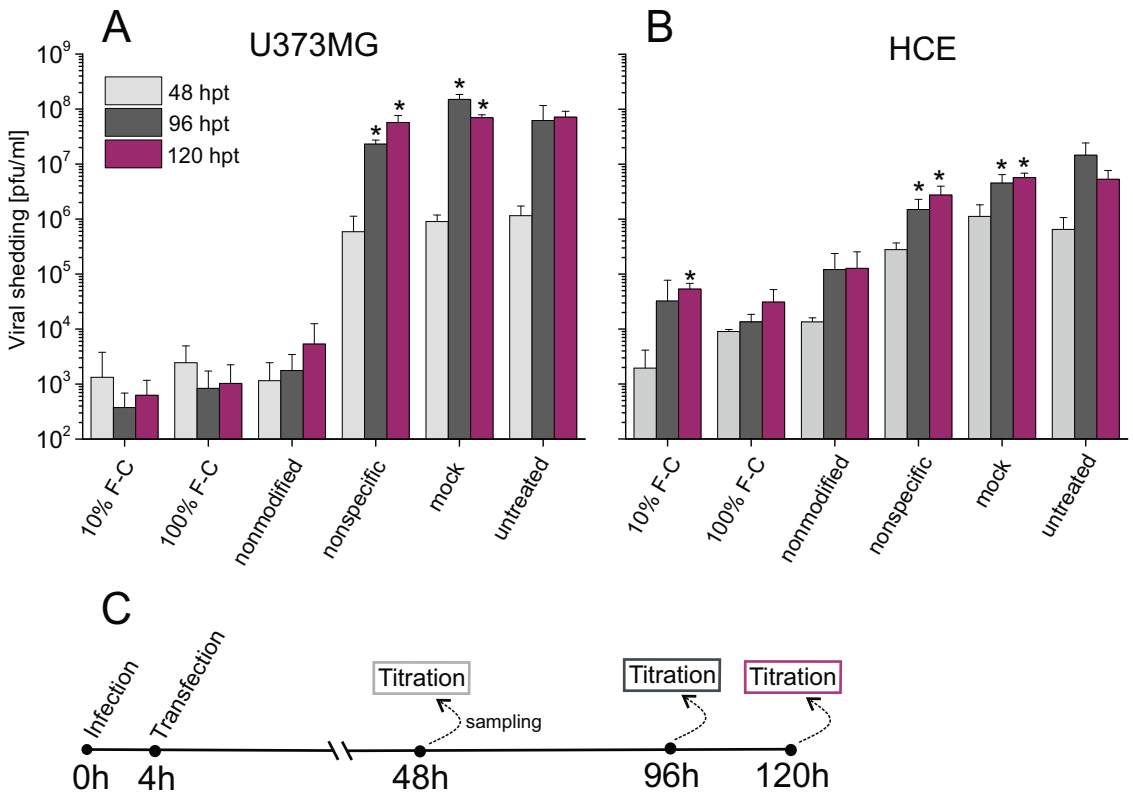

Figure 8 – Sustained inhibition of infection. To examine how long the infection stays inhibited in the therapeutic treatment model, U373MG or HCE cells were infected with 1000 pfu of HSV1-GFP and treated 4 hours post infection with 50nM of the indicated siRNA swarms. The used treatments represented partially modified (10% F-C), fully modified (100% F-C) and nonmodified siRNA swarms. The additional controls used were a nonspecific control siRNA swarm (PET), water transfected (mock) and untreated cells. The viral shedding was quantified from the U373MG **(A)** and HCE **(B)** culture supernatants at 48, 96, and 120 hours post transfection (hpt) (2, 4, and 5 days post infection, respectively). The columns represent the mean+SD of ≥4 samples. The significant changes in viral shedding within each treatment group were calculated in comparison to the earliest timepoint, 48 hpt. The significances found are presented with * ($p \leq 0.05$). **(C)** Experiment flow. The cells were infected followed by transfection after 4 hours (therapeutic model). The viral shedding was quantified by titration 48, 96, and 120 hpt.

Overall, in both of the cell lines the inhibition of viral shedding was high when the cells were treated with HSV-specific siRNA swarms (**Figure 8**). In U373MG cells the viral shedding was three orders of magnitude less (99.9% inhibition efficacy) at 48

hpt and five orders of magnitude less (99.999% inhibition efficacy) at 96 and 120 hpt in cells treated with HSV-specific siRNA swarms compared to untreated cells. Complementing these results, the decrease of viral shedding in HCE cells was two orders of magnitude at 48 hpt and three orders of magnitude at 72 and 96 hpt. Most of the HSV-specific treatments did not show significant changes in viral production within the experiment timeline. The only exception was treatment with 10% F-C in HCE cells, which led to significantly higher ($p \leq 0.05$) viral shedding at 120 hpt compared to that of 48 hpt. The cells treated with the nonspecific siRNA swarms or transfection reagent alone had significantly higher ($p \leq 0.05$) levels of viral shedding at 96 and 120 hpt, than at 48 hpt.

2.2.4 Repeated dosing of antiviral siRNA swarms

To examine the benefits of repeated dosing of siRNA swarms, the cells were transfected again at 48 hours after the initial transfection with the same siRNA swarm or control (**Figure 9**). The first transfection was done 4 hours post infection with 1000 pfu (therapeutic model). The modified siRNA swarms used were 10% F-C and 100% F-C. The nonmodified and nonspecific siRNA swarms, mock-transfected and untreated controls were included as well. For experimental setting, see **Figure 9C**.
In U373MG cells, the viral shedding from the double treated 100% F-C cells was significantly lower ($p \leq 0.05$) than that of the cells treated once. The 10% F-C and nonmodified siRNA swarm treated cells also showed similar trend in the titers, but it did not result in statistical significance. The infected cells treated with the nonspecific siRNA swarm resulted in equal titers, whether transfected once or twice. Complementing the results from U373MG cells, in HCE cells, the 10% F-C and 100% F-C treated cells had a five-fold lower titer when dosed twice compared to those which were dosed only once. The HCE cells treated with nonmodified HSV-targeted or nonspecific siRNA swarm, however, demonstrate an increase in titer in the twice transfected cells compared to those transfected only once.

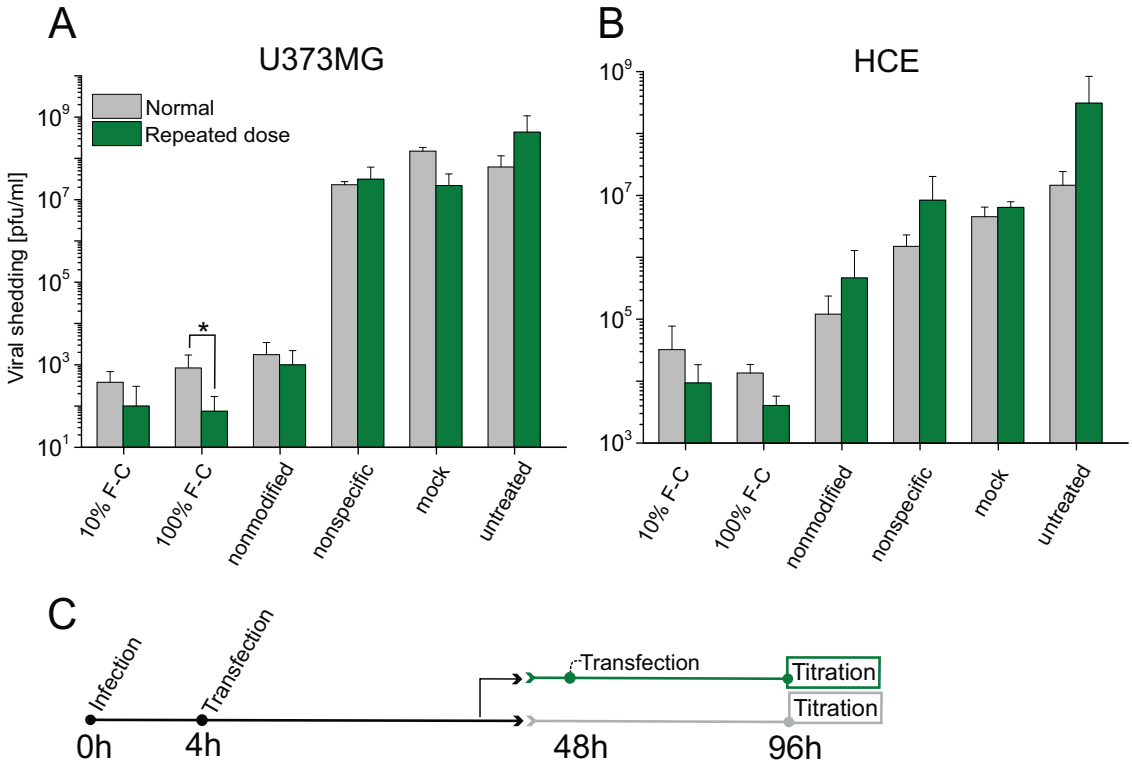

Figure 9 - Repeated dosing of siRNA swarm. To study the possibility of additional dosing of the siRNA swarms, the cells were first infected (1000 pfu) and treated 4 hours afterwards (therapeutic model), followed by another transfection with the same siRNA swarm or control at 48 hours post the initial transfection (green columns, N≥4 per treatment). To control the experiment, cells were also left without the second transfection (grey columns, N≥4 per treatment). At 96 hours, (i.e. 48 hours after the timepoint of the second transfection) the shedding of virus was quantitated by plaque assay (titration) from U373MG **(A)** and HCE cells **(B)**. The used treatments represent partially modified (10% F-C), fully modified (100% F-C) and nonmodified siRNA swarms. The controls used were a nonspecific control siRNA swarm targeting bacterial *lac*-operon, water transfection (mock) and cells left untreated. The p-values were examined between the two settings within each treatment group. All found significances are presented (*, p≤0.05) The columns represent the mean+SD. **(C)** The cells were first infected followed by transfection after four hours. Then, at 48 hpt, the cells were either transfected again with the same siRNA, or not. 48 hours after the second transfection, the supernatants were collected, and viral shedding was quantified.

2.2.5 Antiviral siRNA swarms and viral re-challenge

A therapeutic model (transfection four hours post infection) was utilized to study how the siRNA swarms overcome a viral re-challenge. The cells were infected again 48 hours after initial infection and measured for viral shedding two days after (**Figure 10**). The viral re-challenge was done with a different HSV-1(17+) based fluorescent

virus, HSV1-mCherry, whereas the first infection was done with HSV1-GFP. The siRNA swarms used were the nonspecific, as well as the HSV-specific nonmodified and modified (100% F-C, 10% F-C). As controls, water transfected and untreated cells were used. For experimental design, see **Figure 10D**.

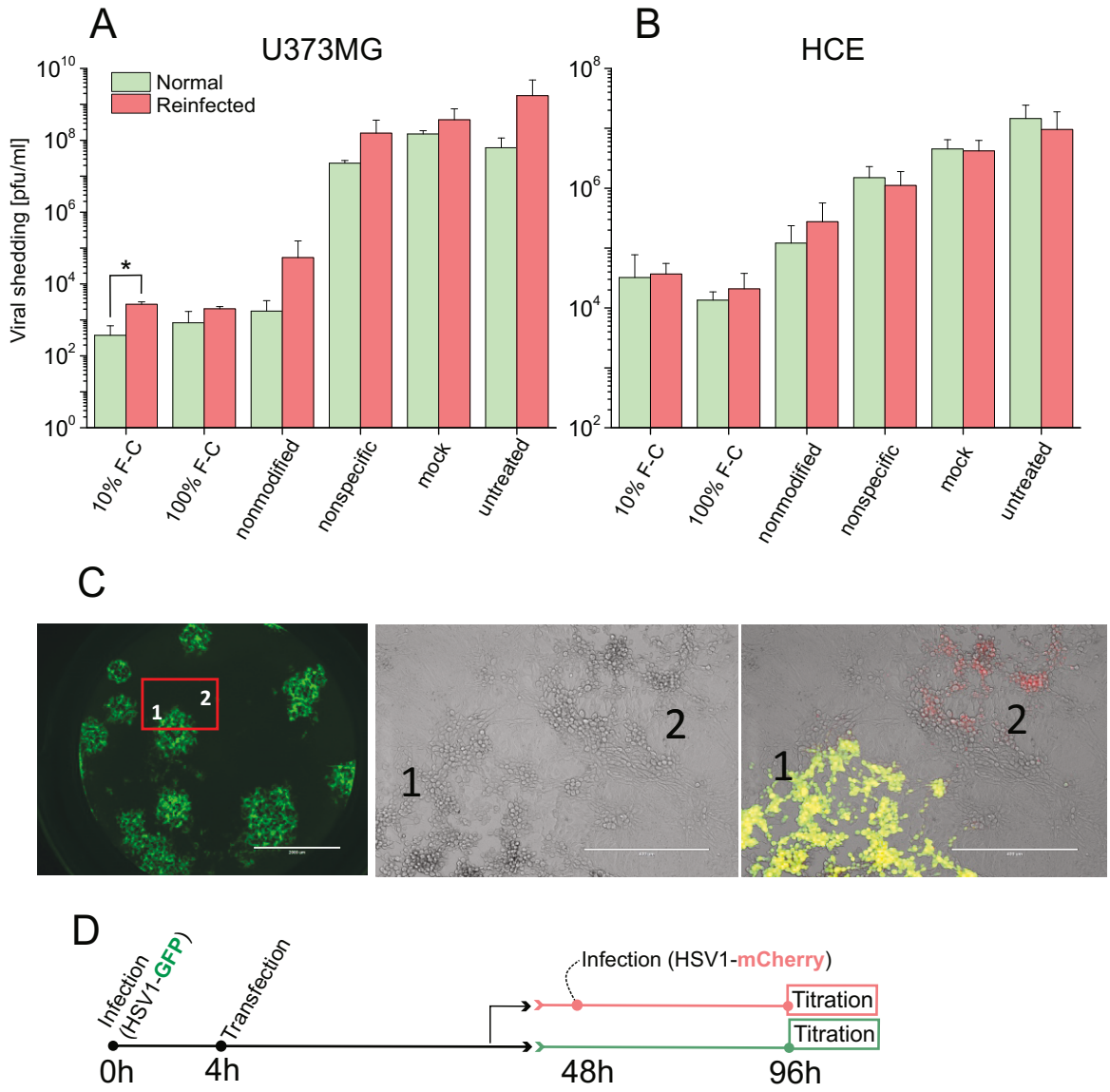

Figure 10 - siRNA swarms and repeated viral challenge. To explore the possibility of the treated cells overcoming a repeat viral challenge, the cells were reinfected (re-challenged, red columns) or not (normal, green columns) at 48 hours post transfection (hpt). Initially, the cells were infected with 1000 pfu of HSV1-GFP, and after 4 hours treated with the indicated siRNA swarms and controls. The experiment initially represented the therapeutic model. The used treatments represented partially modified (10% F-C), fully modified (100% F-C) and nonmodified siRNA swarms. The controls used were a nonspecific control siRNA swarm (targeting a bacterial *lac*-operon sequence), water transfection (mock) and cells left untreated. At 48 hpt, the viral re-challenge was done by infecting the cells with HSV1-mCherry

(1000 pfu). The viral shedding from U373MG **(A)** and HCE cells **(B)** was quantified at 96 hpt (48 hours post viral re-challenge). The statistical differences were examined between the two different settings within each treatment group. All found significances are presented (*, p≤0.05) The columns represent the mean+SD of ≥4 samples. **(C)** Fluorescent imaging of the plaque assay on Vero cells was conducted to prove that the reinfection was successful. As an example, a well with GFP-positive virus plaques (e.g. plaque 1) is shown (left image). In transmitted light, additional plaques were found (middle image), which expressed mCherry (right image, plaque 2). **(D)** Experiment flow. Cells were infected with HSV1-GFP and transfected 4 hours afterwards, representing the therapeutic model. 48h after the initial infection, the cells were re-challenged with HSV1-mCherry, or left as they were. The supernatants were then cultured two days after the re-challenge timepoint.

The anti-HSV siRNA swarm treatments were more antiviral in comparison to nonspecific treatments in both cell lines. In general, the re-challenged cells did not differ statistically from the cells infected only once (**Figure 10A,B**). Even the treated cells, which had yet uninfected cells at the time of re-challenge, did not demonstrate an increase in viral shedding due to viral re-challenge, but had a similar endpoint to those infected only once. This phenomenon was seen in both cell lines. The 10% F-C treated U373MG cells were the only ones that had significantly higher viral shedding when re-challenged (**Figure 10B**). Nevertheless, treatment with the nonmodified anti-HSV siRNA swarm led to a higher increase of titer in both cell lines when re-challenged, compared to the change of the one in cells treated with modified siRNA swarms.

Additionally, fluorescent imaging of the viral titration plaques proved that also HSV1-mCherry infection did happen (**Figure 10C**). This was demonstrated in each of the treatment groups (not shown).

3 Discussion

In this Master's thesis, the major aim was to characterize novel types of antiviral siRNA swarms with 2'-fluoro-modified nucleotides. The initial characterization was done by studying cytotoxicity (**Figure 4**) and host responses **(Figure 6**) induced by the modified siRNA swarms, as well as testing the modified siRNA swarms for antiviral activity (**Figure 5**) in the previously published prophylactic model.

Once the initial characterization of the modified siRNA swarms was done, the therapeutic characteristics of the siRNA swarms were studied further. In addition to the prophylactic model, a therapeutic model, where siRNA swarms are administered after the infection, was included. First, the dose-response relationship between the siRNA swarms and inhibition of viral shedding in these two models was studied **(Table 5** and **Table 6**). As it was clear that therapeutic treatment was successful with siRNA swarms, the infection progress in the therapeutic model was followed for multiple days to find out if the antiviral activity would be sustained for longer than the previously studied interval (**Figure 8**). Subsequently, the ability of the siRNA swarms to overcome a viral re-challenge was studied (**Figure 10**), as well as the regimen of repeated dosing of the siRNA swarm (**Figure 9**). Additionally, the prolonged duration of activity, suggested to result from 2'-fluoro-modifications (Janas et al., 2019; Manoharan et al., 2011), was assessed (**Figure 7**).

The modifications chosen for the studies were fluoro-modifications in the 2'-position of the RNA ribose backbone. The relatively widely studied 2'-fluoro-modifications are proposed to have benefits such as longer stability *in vitro* and enhanced bioavailability *in vivo*, as well as lead to reduction of innate stimulation in comparison to regular siRNA (Fucini et al., 2012; Manoharan et al., 2011). In short, they should have improved qualities compared to nonmodified siRNAs. Importantly, the 2'-fluoro-monomers do not appear to incorporate into cellular DNA or RNA, and are not genotoxic (Janas et al., 2016) or carcinogenic (Janas et al., 2018), which supports the potential of future clinical use of the modified siRNA swarms. Additionally, a deoxy-2'-fluoro-uridine derivative (Sofosbuvir), which has a deoxy-2'-fluoro-uridine metabolite, is already in clinical antiviral use (Kirby et al., 2015). Hence, the incorporation of 2'-fluoro-modifications can be assumed not to lead to major additional difficulties along the pipeline.

The modified siRNA swarms used for the studies represented sequences with all or a part of adenosine (A), cytidine (C) or uridine (U) nucleotides modified in the antisense strand, which is suggested to be the superior strand for antiviral siRNAs (Schubert et al., 2007). Guanosine (G) modified and multi-modified siRNA swarms were omitted due to problems in swarm synthesis and a decrease in yield, respectively (Levanova et al., manuscript). The modification-of-choice was a substitution of the 2´-hydroxyl group to a fluoro-group on the ribose, resulting in only a minimal difference in the molecular weight (F: 18.998403 g/mol, OH: 17.008 g/mol), which can be assumed not to significantly affect concentration calculations of 25 bp long dsRNA molecules. These dsRNA molecules have an approximate molecular weight is 680 g/mol per base pair. The calculations regarding 100% F-C modified siRNA swarm would be affected the most, since the UL29 target sequence has a total of 185 nucleotides (nts) of C in the 653 nt target. In contrast, the sequence has 113 A nts and 128 U nts. If the 3'- and 5'- overhangs of the individual siRNAs in the swarm are considered, the resulting maximal difference in the concentration would be only approximately 1 pmol/µl, which can be considered insignificant for the purposes of this Master's thesis.

3.1 The 2'-fluoro-modifications are well tolerated *in vitro*

The cell lines chosen for the studies were U373MG and HCE, which represent the natural target tissues of HSV-1, in order to provide translational results from the *in vitro* studies. U373MG has been previously used for siRNA swarm studies (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012), whereas HCE has not. HCE is, however, assumed to provide highly translational results for treatment of corneal diseases (Toropainen et al., 2001), and was thus an evident choice for use. After all, siRNA swarms could provide a new approach for treating herpes keratitis, which currently lacks a reliable treatment.

The transfectability of U373MG had previously been shown by Romanovskaya et al. (2012). The transfectability of HCE was checked utilizing a fluorescent siRNA (not shown). Additionally, the transfectability was confirmed by the toxic effect caused by the cytotoxic control, 88 bp, which took place in both of the cell types (**Figure 4**).

3.1.1 The transfection reagent is the likely reason for any siRNA swarm induced cytotoxicity

The transfection reagent used was Lipofectamine RNAiMAX, which is optimal for dsRNA transfection. The reagent is shown to demonstrate relatively low cytotoxicity combined with relatively high transfection efficacy in comparison to other common transfection reagents (Wang et al., 2018). However, RNAiMAX is a probable reason for the majority of the detected decrease in viability, since the reagent alone leads to similar values of cellular viability as transfection with siRNA, whether modified or not (**Figure 4**). The fact that the toxicity levels elicited by double-stranded oligonucleotides is delivery method dependent, regardless of 2'-fluoro-modifications, is supported by literature (Janas et al., 2017). Hence, the most important control for the cytotoxicity studies is so called water or mock transfection, reflecting the cytotoxic effect of the transfection reagent alone.

The cytotoxicity of the treatments was studied with a luminescent assay measuring levels of cellular adenosine triphosphate (ATP) and thus viability of the cells (Romanovskaya et al., 2012; Turunen et al., 2016). Overall, none of the modified siRNA swarms were toxic in either of the cell lines at 50nM, since their relative toxicity is very comparable to that of the cells treated with transfection reagent alone

(**Figure 4A** and **Figure 4B**). Also, as demonstrated by **Figure 4C** and **Figure 4D**, increasing the concentration up to 150 nM has no effect on modified or nonmodified siRNA swarm tolerability in either of the cell types. Thus, the CC_{50} (half-maximal cytotoxicity value) of all modified siRNA swarms must be higher than 150 nM (0.150 µM). The concentration was not increased more since the amount of modified siRNA swarms for use was limited. However, the relationship of the nonmodified siRNA swarm concentration to cytotoxicity has been studied with concentrations up to 500 nM, and even then, no toxic effect was detected (Paavilainen, 2017). Hence, it can be assumed that the level of cytotoxicity will remain similar throughout a larger concentration range but remains to be defined for modified siRNA swarms. However, as monomers, the CC_{50} of the different 2'-fluoro-modified nucleotides has been tested. Then, the CC_{50} varied between 43 and 250 µM depending on the medium and cell type, with surprisingly high variations between different nucleotides demonstrating slightly better tolerability of pyrimidine than purine monomers (Janas et al., 2019). Reaching these concentrations in *in vitro* experiments is unlikely, if even possible. Even in *in vivo* experiments with siRNA swarms, antiviral efficacy was demonstrated with 25 µM (Paavilainen et al., 2017). Moreover, 25 µM was effective even without transfection reagent, so the actual required antiviral dose for *in vivo* use can be estimated to be even lower, if a suitable transfection reagent were to be found. Nevertheless, *in vitro* applications requiring micromolar concentrations are unlikely, especially since the calculated EC_{50}-values for the modified siRNA swarms are well under 5 nM even in the less responsive cell line (**Table 6**).

At the concentration used (50 nM; 0.05 µM), the modified siRNA swarms do not differ in tolerability from the nonmodified siRNA swarms in U373MG cells. However, in comparison to the nonmodified siRNA swarm, some modification types lead to increased cytotoxicity in HCE cells. Both F-C-modified and the fully F-U-modified lead to significantly lower levels of relative viability than their nonmodified counterpart. In this sense, HCE cells are more sensitive to the modifications in siRNAs, and especially those in pyrimidines (**Figure 4B**).

3.1.2 The magnitude of the type I innate response depends on the nucleotide modified

The innate host responses, which were studied at 48 hpt by mRNA analysis, were represented by type I interferon expression (IFN-β), and three different interferon stimulatory genes (MxA, MxB, and ISG54). As for U373MG cells, all types of modifications were studied, but for HCE, only adenosine modified were studied. The reasoning to choose adenosine modified siRNA swarms for the innate immunity studies in HCE cells was related to the slightly elevated cytotoxicity levels of F-C and F-U modified siRNA swarms. The studied host mRNA expressions were normalized to GAPDH, a housekeeping gene commonly used for normalization of quantitative gene expression data. Since GAPDH values can vary between tissue types, but not within a tissue, it is a very reliable method to normalize *in vitro* data for intra-cell line comparisons (Barber et al., 2005). Direct comparisons between two cell types, should however, be assessed more carefully.

Table 7 - Host responses to the treatments at 48 hpt

				siRNA swarms								
						Modified siRNA swarms						
		Baseline	Transfection reagent	Nonspecific	Nonmodified	10% F-A	10% F-U	10% F-C	100% F-A	100% F-U	100% F-C	88 bp dsRNA
U373MG	*IFN-β*	P	+	+	+	+	+	+	+	+	+	+/++
	ISG54	P	++	++	++	++	++	++	++	++	+++	+++
	MxA	P	++	++	++	++	++	++	++	++	++	++
	MxB	P	+	+	+	+	+	+	+	+	+/++	+/++
HCE	*IFN-β*	P	++	++	++	++	ND	ND	+/++	ND	ND	+++
	ISG54	P	+/++	+	+	++	ND	ND	++	ND	ND	+++
	MxA	P	++	++	++	++	ND	ND	++	ND	ND	++
	MxB	P	++	++	++	++	ND	ND	++	ND	ND	++

P *positive expression,* ND *not determined, +/++/+++ less than 10-fold difference / over 10-fold increase / over 100-fold increase in relative mRNA expression compared to the baseline of untreated cells*

In addition to causing the above-mentioned slight cytotoxicity, the transfection reagent (Lipofectamine RNAiMAX) induces host innate responses and antiviral

efficacy both higher than those of untreated cells in both cell types studied **(Figure 5, Figure 6** and **Table *7*)**. Overall, the modified siRNA swarms, and other siRNA swarm treatments (nonmodified and nonspecific) induce similar levels of host response mRNAs in both cell types than the transfection reagent alone does **(Table *7*).** This result complements the results of the cytotoxicity studies and supports previous literature on the matter that the host responses to siRNAs are highly dependent on the delivery method (Nguyen et al., 2009).

Naturally, slight exceptions were found. Most noticeably, 100% F-C induces significantly higher IFN-β and ISG54 responses in U373MG cells than the nonmodified counterpart (**Figure 6A** and **Figure 6C**). Additionally, 100% F-A induces lower levels (nonsignificant) of IFN-β expression, than the nonmodified siRNA swarm in both cell types (**Figure 6A** and **Figure 6B**). Hence, the 100% F-A and 100% F-C differ in this fashion from the rest of the modified siRNA swarms. Some sequence motifs can cause elevated type I interferon response (Judge et al., 2005; Meng and Lu, 2017), but can't be the reason in this case, since both 100% F-A and 100% F-C are derived from the same sequence. That, however, can be the cause of host response differences between different types of swarms, such as the slight difference in IFN-β levels between those induced by the nonspecific and UL29-targeted nonmodified swarms (**Figure 6A** and **Figure 6B**). The suggested, slight reduction of immune stimulation caused by adenosine 2'-fluoro-modifications is supported by literature (Fucini et al., 2012; Meng and Lu, 2017). The results from this study support also those of (Fucini et al., 2012) and (Shin et al., 2007), who showed that cytidine and pyrimidine modifications, respectively, were ineffective in decreasing immune stimulation of the host. Furthermore, in this study the 100% F-C and 100% F-U, representing maximum modifications of pyrimidines, actually highly differ from each other in the respect of host responses, which suggests that evaluating the effect of modified siRNA swarms as groups of purine or pyrimidine should be avoided, if possible.

Nonetheless, based on these host response results, the 2'-fluoro adenosine modifications would seem to be the best choice for therapeutic use, if no additional immune activation is desired. Considering future therapeutic use of siRNA swarms against HSV, low or neutral immune stimulation should be preferred since concomitant treatment with interferons might be realistic regarding for example

herpes keratitis (Minkovitz and Pepose, 1995; Wilhelmus, 2015), and excess interferon levels can result in undesired cytotoxicity, apoptosis or other adverse effects. Still, in some antiviral applications against HSV, or other viruses, immune stimulation of type I interferons caused by siRNAs can be beneficial and support the sequence specific activity of a siRNA drug (Nguyen et al., 2009; Schlee et al., 2006). In that case, the 100% F-C siRNA swarm is the superior candidate. Altogether, understanding and being able to control the host responses to siRNA swarms is of high importance when considering therapeutic use. The importance of controlling and understanding the immune response in treatment of HSV is also indirectly demonstrated by the current pipeline, of which the majority is focused on immunomodulation (**Table *3***).

Because ISG54 and IFN-β were able to distinguish differences between different types of siRNA swarms, they should be used also in the future for RNA treatment characterization. On the contrary, MxA and MxB profiles do not enable distinguishing of any differences between treatment groups. It could, however, be that the timepoint of 48 hpt is too late for determination of these responses. In the future, earlier timepoints should be studied, since in U373MG cells the peak in ISG54 and IFN-β responses induced by siRNA swarm, has been detected at 24 hpt (Romanovskaya et al., 2012). The study of IFN-α would also be of high interest, since it is one of the main cytokines induced by immunostimulatory RNAs (Meng and Lu, 2017).

The similar-to-RNAiMAX innate host responses and cytotoxicity profile indicate that antiviral efficacy detected after treatment with the modified siRNA swarms is sequence specific. 100% F-C is the only modified siRNA swarm that can possess some antiviral efficacy derived from stimulation of innate immunity responses, however, the stimulation of innate responses and the cytotoxicity are not nearly as high as for 88 bp dsRNA, whose antiviral efficacy is merely due to high cytotoxicity (**Figure 4**) and stimulation of innate responses (**Figure 6**). As a conclusion, all the HSV-targeted antiviral swarms tested are sequence specific. Nevertheless, the importance of the nonspecific control is high. Additionally, the 10%-modified siRNA swarms are an important control, since they will help to elaborate the effect of modifications on antiviral activity, since they provide results presumably between the nonmodified and the 100%-modified siRNA swarms.

3.2 Antiviral properties of modified siRNA swarms

3.2.1 The modified siRNA swarms are efficient as antivirals

All the studied modified siRNA swarms target the same sequence within the UL29 gene of HSV-1. The protein UL29 is coding for, ICP8, an essential protein for viral DNA synthesis (de Bruyn Kops and Knipe, 1988), and thus with silenced UL29 expression, the virus is unable to replicate. Altogether, for studying the effect of modifications on the characteristics of siRNA swarms, UL29 is most definitely the target of choice, since it is currently the most extensively studied and promising siRNA swarm target (Paavilainen et al., 2016). Nevertheless, it would be highly interesting to study other viral targets as well. For example, an effect against establishment of latency might be possible, but requires more complex settings.

The antiviral efficacy of the modified UL29 siRNA swarms was first assessed using the prophylactic model, which has been previously utilized in siRNA swarm studies *in vitro* (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012). The antiviral efficacy was measured by viral titration and RT-qPCR analysis of a viral γ-gene's (VP16) mRNA levels (**Figure 5**). By both measures, the virus production decreased significantly when the cells were treated with UL29-specific siRNA swarms in comparison to treatment with a nonspecific siRNA swarm, verifying the sequence-specificity of the UL29 siRNA swarms used. The VP16 RT-qPCR results indicate that the cascade-like gene-expression of HSV-1 is discontinued due to silencing of UL29-mRNA, and the titration results confirm that the UL29-specific silencing leads to a decreased number of infectious viruses produced and shed from the infected cell culture. The results from the titration and RT-qPCR correlated extremely well, and thus in further studies, titration was utilized alone for quantifying the antiviral response, since it is more straightforward and does not require as many steps of specimen preparation as RT-qPCR. Moreover, titration quantifies replication competent viruses and when applicable is considered the golden standard to analyze viral amounts.

Based on the results, the antiviral efficacy of the UL29-specific siRNA swarms is at excellent level of five orders of magnitude in U373MG cells and three orders of magnitude in HCE cells in comparison to the untreated cells (**Figure 5**). U373MG cells have also previously proven the most responsive cell line for antiviral siRNA

swarm studies, but the reported change of viral titer due to treatment with UL29-specific swarms has been slightly lower at approximately three to four orders of magnitude (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012).

As anticipated based on the slightly higher host response levels, the antiviral efficacy of 100% F-C was significantly higher than that of the nonmodified siRNA swarm (**Figure *5*A**). Surprisingly, the 100% F-U was significantly less antiviral than the nonmodified siRNA swarm. The 100% F-A, then again, demonstrated similar antiviral activity to the nonmodified siRNA swarm, which combined with the minimal induction of innate responses, makes the 100% F-A siRNA swarm promising lead (**Figure 6**).

Additionally, the statistically indifferent antiviral properties between the bacterial lac-operon targeting siRNA swarm and GFP-targeting siRNA swarm (**Figure 5B**) indicate that targeting non-essential viral inserts does not interfere with viral production, since HSV1-GFP has a GFP-insert in its genome. This result justifies the use of the GFP-siRNA swarm as a nonspecific control siRNA swarm, even though it has a target in the test virus genome.

3.2.2 The antiviral activity of siRNA swarms is dose-responsive

In the dose-response studies, both the therapeutic model and the prophylactic model were used. The therapeutic model is known to be efficient *in vitro* with single site siRNAs (Backman, 2014; Yuan et al., 2005; Zheng et al., 2004), but was not before demonstrated efficient with siRNA swarms *in vitro*. Nevertheless, the siRNA swarms have already shown therapeutic antiviral efficacy *in vivo* (Paavilainen et al., 2017).

Based on the results of the dose-response study, therapeutic treatment was almost as effective as prophylactic treatment, but as expected, led to slightly lower maximum efficacy values (**Table 5** and **Table 6**). The preexisting elevated innate immunity levels due to siRNA transfection (**Table 7** and **Figure 6**) provide the cells an advantage against the incoming virus in the prophylactic model, whereas in the therapeutic model, the cells are more susceptible upon infection. Interestingly, in HCE cells, the siRNA swarms exhibited higher potency (EC_{50}) in the therapeutic model than in the prophylactic model. The elevated potency might be due to

enhanced transfection efficacy to pre-infected cells, or that as the transfection of viral transcripts is already initiated at the timepoint of transfection, the siRNAs find their targets faster, and the potential degradation of the active siRNA has less significance. In the therapeutic model the antiviral activity may be less sequence specific than in the prophylactic model. The reasons might lie in innate responses. The complex interactions between exogenous RNA and the virus in the cell were unfortunately not within the scope of this Master's thesis. Data already exists the on interactions of virus and the innate response to siRNAs in the prophylactic model (Paavilainen et al., 2016; Paavilainen et al., 2015), but the cell lines did not include HCE and the results from the prophylactic model may not be extrapolated to therapeutic model as such. The hypothesis is nevertheless supported by the larger decrease of viral titer by treatment with the nonspecific siRNA swarm in the therapeutic model, than in the prophylactic model, indicating that elevated levels of non-sequence-specific inhibition of viral replication is present in the therapeutic model. The treatment is in any case sequence specific as the difference in viral shedding is significant between nonspecific and specific treatment. The hypothesis is also supported by an *in vivo* study of corneal infection and subsequent corneal administration of siRNA swarms, where also the nonspecific siRNA swarm exhibited significant antiviral efficacy (Paavilainen et al., 2017).

In general, the results from the dose-response study (**Table 5** and **Table 6**) reveal higher antiviral potency of siRNA swarms harboring any 2'-fluoro-modifications, which could translate to a lower required dose in *in vivo* use. The resulting EC_{50}-values (HCE cells; 2-4 nM), were of a different range than those found from literature. For example, antiviral siRNAs against Hepatitis C reached an EC_{50} of 0.4 nM (Moon et al., 2016) and those against coxsackievirus 3B approximately 0.1 nM (Schubert et al., 2007). And, as a different example, a double stranded 2'-fluoro-modified siRNA, for applications not related to antiviral research, reached EC_{50}-values of <0.1 nM (Haringsma et al., 2012). Nevertheless, in this study, the EC_{50} -values from the more responsive cell line, U373MG, were not determinable due to efficacy of over 80% even at the lowest concentration (0.41 nM) used, which suggests a comparable, sub-nanomolar EC_{50}-value.

Notably, the therapeutic and prophylactic maximum efficacies of the UL29-targeted siRNA swarms, whether modified or not, are higher than maximum efficacies found

from the literature (Schubert et al., 2007; Zheng et al., 2004). To specify, the siRNA swarms are not able to totally cure the infection, although it might be suggested by the 100% maximum efficacy in U373MG (**Table 5**). Still, some minimal virus shedding was detectable in the culture supernatants, and the actual maximum efficacy was rounded up from >99.99%. To current knowledge, full therapeutic antiviral efficacy with siRNAs *in vitro* can be reached with repeated doses only (Saulnier et al., 2006).

The relationship between siRNA swarm concentration and inhibition of HSV replication is dose-responsive, and highly cell line dependent. The dose mainly used throughout the Master's thesis (50nM) is relevant for both of the cell lines for such *in vitro* studies seeking maximum antiviral efficacy, since at 33.3 nM the maximum response is detected in both cell lines. Hence, a dose close to 33.3 nM, such as 50 nM allows for maximum response with less RNA than the 100 nM dose traditionally used in siRNA swarm studies. For future siRNA swarm applications, the assessed dose-response relationship provides highly important knowledge, whether the siRNA swarms are modified or not, and whether they are used for antiviral purposes or other applications. However, the dose-response study in U373MG cells should be completed until the point of no antiviral response is reached, and the study could be repeated with swarms harboring other modifications than those in adenosine as well.

3.2.3 Transfected siRNA swarms remain antiviral in cells for at least four days

One of the major reasons, why 2'-fluoro-modifications of the siRNA swarms were considered, was to enhance the proposed stability of the siRNA *in vitro* and *in vivo* (Manoharan et al., 2011). Recently, pyrimidine modified siRNA swarms were proven more stable to RNase A compared to nonmodified nucleotides (Levanova et al., manuscript). However, cellular stability of modified or nonmodified siRNA swarms was not yet studied. Hence, the stability of active, antiviral siRNA in cells was studied by transfecting cells and waiting for four to 96 hours before infecting. The cells are always washed after transfection, suggesting that the all active siRNA is intracellular. For the study, a short single site UL29-targeting siRNA, UL29.2 was also included for comparisons (Palliser et al., 2006). In all of the timepoints studied, all of the HSV-targeted siRNAs, both single-site and swarm, demonstrated antiviral activity of over

99.9% compared to the untreated cells, as well as significantly lower levels of viral shedding than those resulting from nonspecific treatment in almost all groups. In HCE cells, some of the treatments did not reach significance against the nonspecific siRNA swarm treatment, but that is likely to result from the low sample size, rather than lack of sequence specific efficacy.

Notably, the timepoint of infection at 24 hpt appeared to be the best regarding antiviral efficacy, since a majority of the HSV-specific treatments led to total inhibition of HSV-replication (**Figure 7**). That, however, is not the most relevant timepoint for implications of acute infection, since the patient would need to recognize the reactivation of herpes extremely early. Nevertheless, the high prophylactic efficacy of an anti-HSV drug, even for use after symptoms occur, is important. In clinical herpes infection, the virus spreads from cell to cell, and thus multiple phases of infection are ongoing simultaneously. Hence, even given well after herpes infection symptoms arise, the antiviral siRNA swarm could efficiently protect neighboring cells for multiple days, and thus prevent the spread of infection. Simultaneously, the siRNA swarm will decrease the viral load from cells already infected and thus inhibit transmission of infectious virus (**Table 5**, **Table 6, Figure 8, Figure 10**). Additionally, for prophylactic treatment of recurrent HSV, such as is required in treatment of herpes keratitis, the long duration of antiviral efficacy is highly encouraging. Potentially, with long term use, recurrences could be prevented altogether, as suggested by *in vitro* results from 24 hpt (**Figure 7**). To point out, these results are derived from *in vitro* studies, and thus do not necessarily translate to *in vivo* nor clinical use.

Interestingly, since the cells proliferated after transfection, as confirmed by microscopy, and still no evident difference in antiviral efficacy was detected between the earlier and later timepoints, the results suggest that the siRNA ends up also in the daughter cells. If it wouldn't, the non-transfected cells would have produced detectable virus. However, challenging the interpretation of the results, the confluency of the cells affects their susceptibly to HSV.

Overall, in the timepoints studied, no evident, systematic differences between the stability of the nonmodified and modified siRNA swarms could be detected. Therefore, the timepoints studied need to be continued further in order to see the possible prolonged stability and subsequent activity caused by incorporated

modifications. Normally, siRNAs lose *in vitro* efficacy in approximately a week (Bartlett and Davis, 2006), which could be the desired timespan in the repeated experiment. Longer experiments, however, would require a more complex setting, and thus require additional optimizations. Changes in current protocol, such as using lower siRNA concentrations or 100% confluent cell layers upon transfection, could also provide the needed result.

Regardless, remaining active in cells for four days with equal or better antiviral activity than that of the single site siRNA, and furthermore without losing antiviral efficacy (**Figure 7**), revealed yet another favorable aspect of the siRNA swarm approach. Interestingly, the same single site siRNA used here remained antivirally active for 9 days *in vivo* (Palliser et al., 2006), which actually suggests similar, or even better indications for use of the siRNA swarms *in vivo*.

3.2.4 One dose of siRNA swarm controls HSV-1 infection for at least five days

In the study for sustained inhibition of infection (**Figure 8**) supernatants of cells treated with siRNA swarms, with and without fluoro-modifications, were quantified for replicative virus at 48, 96, and 120 hpt utilizing the therapeutic model.

Surprisingly, infection was almost totally suppressed in each anti-HSV siRNA swarm treated group throughout the three-day period (48–120 h) with no significant increase in viral production. (The only exception was the 10% F-C modified at 120 hpt in HCE cells.) The non-specific treatment did not suppress the infection, as the viral shedding increased significantly in the three-day period ($p \leq 0.05$) in both cell lines. Since the viral shedding from the controls increased, whereas that of the anti-HSV siRNA swarm treated didn't, the antiviral efficacy was relatively higher at the later timepoints in U373MG cells. Normally, the viral shedding is quantified at 48 hpt, but these results indicate that the antiviral response would be favorable to measure at later timepoints.

In HCE cells, treatment with fully modified siRNA swarm (100% F-C) was able to suppress the infection better than the non- and partially (10% F-C) modified siRNA swarms. Additionally, in U373MG cells the fully and partially modified swarms clearly suppressed the infection better than the nonmodified swarm. Because of these results, the durability of action of the 2'-fluoro-modified siRNA swarms appears better

than that of the nonmodified siRNA swarms. Either the modified siRNA swarms remain active in the cells for longer and at higher concentrations (e.g. by avoiding nucleases), or the primary inhibition of viral replication is so much more potent, that the relative escape from the treatment is negligible.

Whether the infection of anti-HSV siRNA swarm treated cells would eventually reach the levels of untreated cells remains to be studied. Again, the timepoints should have been continued further to see whether the infection would begin to spread, and if so, when. However, combined with the fact that HSV-1 doesn't develop resistance against siRNA swarm treatment (Paavilainen et al., 2016), the ability to prevent virus growth for five days with one therapeutic dose is highly encouraging for therapeutic applications.

3.2.5 Repeated dosing of siRNA swarms improves antiviral outcome

The repeated dosing was studied by giving another dose of the initial treatment at 48 hours after the initial treatment, which was given four hours after infection (for clarification, see **Figure 9C**). The timepoint was chosen because the viral shedding between 48 and 120 hours was known in the therapeutic setting used (**Figure 8**).

Increased inhibition of viral shedding was detected in the treatment groups treated with HSV-specific siRNA swarms. And, even though in majority of these treatment groups the increased inhibition with a repeated dose was nonsignificant, the results are encouraging. The repeated treatment was indeed possible and demonstrated increased antiviral efficacy compared to treatment with a single dose (**Figure 9**). As previously mentioned, full eradication of virus *in vitro* has been shown only with repeated doses (Saulnier et al., 2006). However, here the dose was repeated only once, raising the question of what would happen with multiple repeats - total eradication of virus, elevated cytotoxicity, or perhaps both.

3.2.6 siRNA swarms can resist a viral re-challenge

Whether one dose of siRNA swarm would be able to overcome a viral re-challenge was assessed by utilizing the therapeutic setting, after which, the cells were re-infected at 48 hpt. The second infection was done with virus with a different

fluorescent protein, HSV1-mCherry, which enabled confirming that the re-infection indeed happened.

In general, the results support that the antiviral siRNA swarms, both modified and nonmodified can treat an infection and prevent a second, subsequent infection (**Figure 10**). In the cells treated with antiviral siRNA swarms, the levels of viral shedding did not increase to the extent they could have but remained like their own corresponding once-infected control. Importantly, the success of the second infection was confirmed by fluorescent live microscopy (**Figure 10C**). Hence, the treatment efficacy remained, indicating that the antisense siRNA strand was not decayed, or that there was plenty of active siRNA left after the first silencing round. The reasons for the extended activity might be related to siRNA swarms being Dicer-substrate dsRNA with elevated silencing potency (Kim et al., 2005) or their other miRNA-like features (Lam et al., 2015).

For future confirmation of the phenomenon, the setting should be adjusted so, that the reinfection would lead to more evident increase in viral shedding in the control groups. It can be simply reached with lower amount of virus in the first infection (e.g. 100 pfu). Such a setting would enable more direct comparisons between the specific and nonspecific treatments. Yet, even though the results need more clarification, they highly indicate that treatment with an antiviral siRNA swarm can suppress subsequent viral infection after eliminating the first one.

3.3 Conclusions and future aspects for siRNA swarm studies

The results of this Master's thesis provide many valuable pieces of information regarding modifications in the siRNA swarms, and therapeutic dosing of siRNA swarms *in vitro*. In general, the studies support the use of modified swarms in further research, since they were well tolerated with no exceptions (**Figure 4**), did not demonstrate alarming changes in host response levels (**Figure 6**), and were antiviral in various settings (**Figure 5, Figure 7, Figure 8, Figure 9, Figure 10**). The modifications appeared to lead to superior results in antiviral activity (**Table 6**), warranting further investigation. The modified siRNA swarms may also be advantageous in the sense of manipulation of host innate responses, which seem to elevate due to cytidine modifications, and slightly decrease due to adenosine modifications. In the studies conducted to learn more about siRNA swarm stability and duration of action (**Figure 7**) as well as the siRNA swarms' prolonged ability to suppress infection (**Figure 8, Figure 10**), the modified siRNA swarms were not clearly superior in comparison to the nonmodified siRNA swarms. However, the modified siRNA swarms appeared to have increased antiviral efficacy upon re-dosing (**Figure 9**), whereas the nonmodified didn't. Overall, depending on the needs of the subsequent study lineages of research on siRNA swarms, the modifications may be beneficial to incorporate into the siRNA swarm.

Interestingly, some of the modified siRNA swarms have demonstrated superior stability against nucleases in comparison to the nonmodified siRNA swarm. Thus, even though the stability difference could not be established by cellular means *in vitro* (**Figure 7**), the stability studies would be reasonable and relevant to continue *in vivo,* especially since elevated *in vivo* stability due to incorporated 2'-fluoro-nucleotides is suggested by previous research (Manoharan et al., 2011). Nevertheless, it seems that even regular siRNA swarms have higher stability than single site siRNAs, which suggests extensive stability for the modified siRNA swarms.

If modifications can be utilized for further research, the adenosine modified siRNA swarms are a clear lead and the most promising candidate. The 100% adenosine modified siRNA swarm (100% F-A) led to slightly lower levels of host innate response combined with elevated antiviral potency in comparison to the nonmodified

siRNA swarm. The results indicate that 100% F-A indeed was superior to the nonmodified siRNA swarms. Yet, not all modified siRNA swarms were studied to the same extent. Considering that they had such differing characteristics when their comparison was possible (**Figure 4**, **Figure 5**, **Figure 6**), further studies might reveal additional interesting characteristics. Unfortunately, it was not possible to utilize all of the modified siRNA swarms in the same experiments in this Master's thesis. Nevertheless, it might be more reasonable to continue studies with the adenosine modified siRNA swarms, than to deepen the knowledge of cytidine modified, which induced elevated innate responses (**Figure 6**), or uridine modified, that actually demonstrated surprisingly low antiviral efficacy (**Figure 5**).

The partially (10%) modified siRNA swarms, in which the presence of the 2'-fluoro-dNTPs was confirmed by mass-spectrometry (Levanova et al., manuscript), were similar in nature to the nonmodified throughout almost all the studies. However, in the dose-response study, partly modified siRNA swarms also led to increased antiviral efficacy and potency. Synthetizing the 10% modified siRNA swarms is more affordable than 100% modified siRNA swarms. It however remains to be studied further whether a small number of modified nucleotides will suffice for the same effects as the fully modified. Overall, an *in vivo* study, at best including all the fully and partly modified siRNA swarms would be very informative. The characteristics of the individual modified siRNA swarms would then be confirmed, and the highly important *in vivo* stability assessed.

As a conclusion, the siRNA swarms offer a very valuable antiviral tool for the future. By covering a large genomic area, the likelihood of resistance is lower, choosing the target is easier and the possibility for adverse off-target effects is diluted. Moreover, based on previous results, siRNA swarms tolerate variability between circulating strains of pathogens (Jiang et al., 2019; Nygårdas et al., 2009; Paavilainen et al., 2016), are efficient *in vivo* (Paavilainen et al., 2017) and do not cause adverse levels of innate response in human cells nor are toxic (Paavilainen et al., 2015; Romanovskaya et al., 2012). Now, in addition to the these previously known characteristics, it is shown that they are therapeutically efficient *in vitro* and suppress viral infection for at least five days (**Figure 8**). Moreover, one therapeutic dose is enough for the cells to even resist a viral re-challenge (**Figure 10)** and the siRNA swarms remain antivirally active in cells for at least four days upon prophylactic

dosing (**Figure 7**). These remarkably promising new results were reached with and without modifications. As previously summarized, the modified siRNA swarms manifested certain considerable benefits over the nonmodified siRNA swarm. Based on the *in vitro* data, the adenosine modifications especially offer an extremely promising lead, if studies with siRNA swarms with incorporated modifications are to be continued. Yet, only *in vivo* data will surely determine the value of 2'-fluoro-modifications as a part of siRNA swarms and confirm if they are essential for future studies leading towards therapeutic applications.

4 Materials and methods

4.1 siRNA swarms and control RNAs used

The RNAs used in this thesis, as well as their target sequences, are described in **Table 8**. Shortly, all of the HSV-specific modified and the nonmodified siRNA swarms target a 653 nucleotide (nt) sequence of the essential UL29 beta-gene of HSV-1, which is the current target of choice for siRNA swarm therapy (Paavilainen et al., 2016). The non-HSV-specific swarms, PET and GFP, target non-viral sequences of pET32b vector and eGFP, respectively, and have inexistent similarities with the HSV-1, human and murine sequences as previously confirmed by Blast. In some experiments a single-site siRNA, UL29.2 (Palliser et al., 2006) was included as a non-swarm siRNA control. For innate immunity and toxicity studies, an 88 bp dsRNA, known for its high cytotoxicity, was utilized as a positive control (Jiang et al., 2011). The modified siRNA swarms had modifications in their antisense strand in either all (100%) or a part (10%) of a chosen nucleotide, which was adenosine (A), cytidine (C) or uridine (U) (**Table 8**).

4.1.1 siRNA swarm synthesis

All the siRNA swarms used, as well as the 88 bp dsRNA, were synthesized by our collaborative research group (dos. Minna Poranen group, Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Finland). The enzymatical synthesis was done as described by Romanovskaya et al. (2012), resulting in 25 bp long double-stranded siRNA molecules (with 2 nucleotide overhangs in 3´ and 5´ ends) of the targeted sequence. The exact enzymatical synthesis of the modified siRNA swarms, with incorporated 2′-fluoro-modified nucleotides on the antisense strand is described in (Levanova et al., manuscript). The presence of the modified nucleotides in the partially modified swarms (10% F-N) was verified by mass spectrometry (Levanova et al., manuscript).

Table 8 - RNAs used

RNA	Type	RNA target sequence	Modification		Reference
			nucleotide	%	
10% F-A	siRNA swarm	UL29 gene of HSV, 653 bp sequence *(nucleotides 59302-59954[a])*	A	10	(Paavilainen et al., 2016)
10% F-C			C	10	
10% F-U			U	10	
100% F-A			A	100	
100% F-C			C	100	
100% F-U			U	100	
nonmodified			None		
nonspecific (PET)		pET32b vector lac-repressor, 401 nt sequence[b], *(nucleotides 1630-2030)*	None		This study
nonspecific (GFP)		eGFP, 711 nt sequence	None		(Paavilainen et al., 2016)
UL29.2	single-site siRNA	UL29 gene of HSV, 19 nt sequence *(nucleotides 59931-59949[a])*	None		(Palliser et al., 2006)
88 bp dsRNA	dsRNA	bacteriophage ϕ6 S segment, 88 bp sequence	None		(Jiang et al., 2011)

[a]HSV-1 strain 17+ (Genbank **JN555585.1**)

[b] According to the numbering of the pET32b vector

4.2 Cell lines used

A human glioma cell line, currently reclassified as U251, but here referred as U373MG (ATCC, Manassas, VA, USA) to assure continuity and comparability with earlier research, as well as an immortalized human corneal epithelial cell line (HCE), kindly provided by Arto Urtti (University of Helsinki, University of Kuopio, Finland), were used in the experiments of this Master's thesis. Additionally, viral plaque titration was done with African green monkey cells referred here as Vero cells (ATCC).

Vero cells were maintained in M199 (Biosera, Nuaille, France) medium supplemented with 5% heat inactivated fetal bovine serum (FBS) (Serana, Silicon Valley, CA, USA) and gentamycin, U373MG cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 2mM L-Glutamine (Sigma, Saint Louis, MO, USA), and HCE cells were maintained in DMEM with Hepes (Gibco), supplemented with 7% FBS. All cells were cultured with antibiotics (usually gentamycin), with exception of cells used for transfection. The cells were provided and used in 96-well plates (Corning, NY, US) by collaborative cell culturing facilities with the confluency of 30-40% (HCE), 40-60% (U373MG) or 100% (Vero). The values were chosen based on manufacturers transfection protocol and own observations for optimized antiviral response (data not shown).

4.3 Viruses used

A green fluorescent protein (GFP) -expressing strain of HSV-1, HSV-1(17+)LoxP_{mCMV}GFP (abbreviated HSV1-GFP) (Mattila et al., 2015; Snijder et al., 2012) and a red monomeric fluorescent protein mCherry -expressing strain of HSV-1, HSV1(17+)Lox-CheVP26 (abbreviated HSV1-mCherry) (Sanbaumhüter et al., 2012) were used. Both recombinant viruses were originally made available by prof. Beate Sodeik (MHH Hannover Medical School, Germany). The green fluorescent protein is expressed from a mouse cytomegalovirus (mCMV) promoter and thus expressed continuously in infected cells after the alpha regulatory phase of HSV. The mCherry protein is co-expressed as a fusion construct with Viral Protein 26 (VP26), which is a small capsid protein encoded by UL35, a late gene expressed in the nucleus (Booy et al., 1994; McNabb and Courtney, 1992).

The virus stocks used were propagated in Vero cells and stored in MNT-buffer [20nM MES (Sigma), 100mM Natrium Chloride, 30nM Tris] as described in detail by Romanovskaya et al. (2012).

4.3.1 Fluorescent imaging

The live cell imaging was performed with the EVOS Auto FL (Thermo Fisher Scientific, Waltham, MA, USA) instrument using either GFP or RFP (red fluorescent protein) filters for HSV1-GFP and HSV1-Cherry, respectively.

4.4 Transfection

For transfection, the cells were plated without antibiotics at least 24 hours before transfection. The cells were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's forward transfection protocol, with a slight modification of using a total volume of 100 µl.

In short, the cells were washed once and left with 80 µl of Opti-MEM (Gibco). The RNA dilutions containing 1) Opti-MEM and lipofectamine (97:3) and 2) Opti-MEM and RNA (ratio depending on concentration of synthesized RNA stock) were prepared, combined 1:1 and let incubate for 20 min. The needed amount of RNA stock (synthesized siRNA swarms, or other RNA diluted in mQ-water) was calculated beforehand from the concentration provided with the following formula: $(c_{stock,\ ng/\mu l} / M_{RNA}) \times 1000 = c_{stock,\ pmol/\mu l}$, where $M_{RNA} = M_{average\ molar\ mass\ of\ dsRNA} * bp_{length\ of\ RNA}$. For siRNA swarms, M_{RNA} = 17 000 g/mol (length ≈ 25 bp, molecular weight of dsRNA bp ≈ 680 ng/mol).

Twenty µl of the prepared dilution was pipetted to the 80 µl of Opti-MEM already covering the cells, whereafter the plates were briefly shaken on a shaker. The cells were then incubated (+37°C, 5% CO_2) for four hours before washing thrice with DMEM (incl. Hepes) with 7% FBS followed by incubation with 200 µl of the same medium.

The cells were transfected with 0.041 to 15 pmols of RNA per well depending on the experiment, leading to RNA concentrations of 0.41 to 150 nM, respectively. For water transfections, 1% of the transfection volume was nuclease free water (Thermo Fisher), reflecting the approximate volume of the siRNAs (diluted in water) required for the dilutions.

4.5 Viability assay

The cytotoxicity of the siRNA swarms was evaluated and quantified with a luminescent assay, CellTiter-Glo (Promega, Madison, WI) at 48 hours post transfection, according to manufacturer's protocol and as described previously (Romanovskaya et al., 2012; Turunen et al., 2016). The luminescent signal was quantified with VICTOR Nivo Multimode Plate Reader (Perkin Elmer, Waltham, MA).

4.6 Antiviral assays

The antiviral assays in previous anti-herpes siRNA swarm studies were conducted by infecting the cells with 1000 plaque forming units (pfu) four hours post transfection (hpt), to mimic the low virus transmission in clinical situations (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012). Additionally, to better represent the clinical situation, another antiviral setting, where the cells were infected before transfection, was utilized. This setting is referred as the therapeutic model and the previously published the prophylactic model.

Table 9 - Antiviral assay settings

	Infection post transfection (Prophylactic model)	Transfection post infection (Therapeutic model)
at 0 h	Cells washed 1x with Opti-MEM, transfection	Cells washed 1x with DMEM 2% FBS, infection*
at 1½ h	-	Wash 2x with Opti-MEM, left with 80 µl
at 4 h	Wash 3x with DMEM 2% FBS, infection	Transfection
at 5½ h	Wash 2x and leave with DMEM 7% FBS	-
at 8 h	-	Wash 3x and leave with DMEM 7% FBS
48 to 120 h	*Follow up and sampling at desired timepoints*	

Cells kept and incubated at +37°C 5% CO_2 when not working

In short, the settings used in this Master's thesis represent therapeutic treatment (transfection four hours after infection) and prophylactic treatment (infection four

hours after transfection) (**Table 9**). The settings were modified in some of the dosing experiments to suit different purposes: 1) In the study for active siRNA stability after transfection, the cells were infected at 24, 48, 72 or 96 hpt, which was done as described with the viability assay. 2) In the re-challenge and repeated dosing experiments, 20 µl containing 1000 pfu or lipofectamine-siRNA -complexes, respectively, were added on top of therapeutically treated culture supernatant at 48 hpt.

4.6.1 Infection

Prior to infection, the cells were washed with DMEM (incl. Hepes) supplemented with 2% FBS and gentamycin. The number of washes depended on the setting (see **Table 9**). The viral dilution, always containing 1000 plaque forming units (pfu) in 100 µl, was pipetted onto the cells. For the standard confluencies at the time of transfection, this amount represents 0.06 and 0.29 multiplicity of infection (MOI) values for U373MG and HCE cells, respectively. The viral dilution was calculated beforehand based on the concentration (pfu/ml) of the used stock [pfu_{needed} / $(pfu/ml)_{stock}$ x 1000 = $µl_{of\ stock\ needed}$]. The stock is made from virus shedded from the cells used in virus propagation, which are then stored in MNT buffer (20 mM MES, 100 mM NaCl, 30 mM Tris, pH 7.4). The viral MNT stock, stored in -80°C, was thawed slowly on ice to minimize the loss of viable viruses. After preparation of the virus dilution, it was incubated on the cells for 1-1.5 h shaking on a shaker at +35°C and afterwards washed to remove the unabsorbed viruses with either Opti-MEM or DMEM (incl. Hepes) supplemented with 7% FBS, depending on the setting (**Table 9**).

4.6.2 Collection of samples

At the desired timepoints the supernatant was collected to a parallel 96-well plate (Corning) and stored at -80°C. Immediately after supernatant collection, those cells intended for mRNA analysis by qPCR, were covered with 80 µl of TRI Reagent (Invitrogen) and stored at -80°C.

4.7 Plaque titration of the virus

The viral titers were determined by plaque titration on Vero cells in 96-well plates using 10-fold dilution series.

The medium on the Vero cells was changed to 100 µl of DMEM (incl. Hepes) supplemented with 2% FBS. A 10-fold dilution series of the sample was prepared. For each dilution, the supernatant was mixed thoroughly by pipetting up-and-down. After dilutions, the plates were incubated 1-1.5 h shaking on an orbital shaker at +35°C. After incubation, 100 µl of DMEM (incl. Hepes) supplemented with 7% FBS and 80 mg/l of human immunoglobulin G (IgG), KIOVIG (Baxalta, Wien, Austria), was added on top of the pre-existing medium. After 3 to 4 days of incubation, the cells were fixed and stained.

For fixing, the supernatant was removed and replaced with methanol (+4°C). After 5 minutes in room temperature, the methanol was removed. The cells were let dry in a laminar flow for at least 5 minutes before addition of the crystal violet 0.1% diluted in phosphate buffered saline (PBS), HyClone (GE Healthcare Lifesciences, Marlborough, MA, USA)). The crystal violet was removed, and the cells were rinsed with tap water and let dry for at least 24 h before counting and imaging the plaques (for example of viral plaques, please see **Figure 10C**).

Due to the presence of IgG in the titration medium, the virus can spread only from cell-to-cell, forming infected areas called plaques. The infected cells then detach, leaving visible hole to the cell layer upon fixing. The plaques are then counted and the plaque forming units per ml are calculated. The plaques can be counted with a regular light microscope.

4.8. RNA extraction

The RNA extraction of the cells on the 96-well plates covered by TRI Reagent was done according to the manufacturer's protocol. In short, with chloroform (20% of TRI Reagent volume) and centrifugation, the RNA was separated in to a clear, aqueous removable phase. From that phase, the RNA could be precipitated with isopropanol (50% of TriReagent volume) and ethanol (at least 100% percent of TriReagent volume). The resulting RNA was dissolved into 20 µl nuclease free water and stored at -80°C.

4.9 Reverse transcriptase reaction

The whole cellular RNA from the both infected and non-infected, treated and control-treated samples was processed into complementary DNA (cDNA) in a reverse transcriptase (RT) reaction as previously described (Paavilainen et al., 2016; Paavilainen et al., 2015; Romanovskaya et al., 2012). In short, the samples were pretreated for 30 minutes at +37°C with DNAse (Thermo Fisher Scientific, Waltham, MA) in the presence of RNAse inhibitor (Thermo Fisher Scientific) followed by deactivation of the DNAse with EDTA (Sigma Aldrich, Saint Louis, MO, USA). The RT reaction was conducted using RevertAid H Reverse Transcriptase (Thermo Fisher scientific) with random hexamer primers (Thermo Fisher Scientific), which were annealed 5 min in +70°C, followed by cooling down on ice. The RT-cycle used was 20 min at +37°C, 60 min at +42°C and 10 min at +70°C. After cooling down at +4°c, the resulted cDNA was stored at -80°C.

4.10 Quantitative PCR

Quantitative PCR (qPCR) was performed with a Rotor-Gene Q (Qiagen, Hilden, Germany) instrument utilizing a SYBR Green enzyme and buffer system (Thermo Fisher), as previously described (Nygårdas et al., 2011). The mRNA expression of interferon beta (IFN-β), interferon stimulated gene 54 (ISG54), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), human myxovirus resistance protein A (MxA), human myxovirus resistance protein B (MxB) and HSV viral protein 16 (VP16; alpha-TIF) were quantified using primers listed in **Table 10**.

The housekeeping gene (GAPDH) values of each sample were used for normalizing mRNA expression levels. Quantity standards (calibrators), specifically custom-made for each primer pair, were used. For MxA, the standard has been used in (Yahya et al., 2017), for MxB, the standard is described in Kalke et al. (manuscript in preparation), and the rest are listed in (Paavilainen, 2017).

Table 10 - qPCR primers used

Target		Sequence	Reference
GAPDH[a]	sense	GAG AAG GCT GGG GCT CAT	(Nygårdas et al., 2009)
	antisense	TGC TGA TGA TCT TGA GGC TG	
ISG54[a]	sense	ACT ATC ACA TGG GCC GAC TC	(Romanovskaya et al., 2012)
	antisense	TTT AAC CGT GTC CAC CCT TC	
IFN-β[b]	sense	TCT CCA CGA CAG CTC TTT CCA	(Peri et al., 2008)
	antisense	ACA CTG ACA ATT GCT GCT TCT TTG	
MxA[b]	sense	GAG GAG ATC TTT CAG CAC CTG	Used in (Yahya et al., 2017); sequences in Kalke et al., manuscript in preparation
	antisense	TGG ATG ATC AAA GGG ATG TG	
MxB[a]	sense	GGA AAG CAG CGT CCT TCT CT	Kalke et al., manuscript in preparation
	antisense	ATT CCT TCC AGC AAC AGC CA	
VP16[a]	sense	GCT CCG TTG ACG AAC ATG AA	(Broberg et al., 2003)
	antisense	TTT GAC CCG CGA GAT CCT AT	

[a]*The annealing temperature in the PCR cycle was 55°C*

[b]*The annealing temperature in the PCR cycle was 60°C*

4.11 Statistical analysis

Statistical analysis was done with SPSS Statistics 26.0.0.0. (IBM, Armonk, NY). The normality of the data was assessed both visually and with Shapiro-Wilk test. The statistical significances were all calculated with Mann-Whitney's non-parametric U-test comparing two individual groups. The sigmoidal dose-response curves were fit and EC_{50} values calculated with Origin 2016 (64-bit) b9.3.3.303 (Academic) (OriginLab Corporation, Northampton, MA, USA).

Acknowledgements

First, I would like to acknowledge my supervisors Veijo Hukkanen and Henrik Paavilainen for all the great guidance and advice. Second, I would like to acknowledge Liisa Lund and Marie Nyman for all the extremely valuable help with the lab-work, Marja-Leena Mattila for cell culturing, Laura Kakkola for kind guidance of working in biosafety 2 level laboratories, and Ritva Kajander for teaching me the ways around the virus laboratories. Third, I want to acknowledge Minna Poranen, Alesia Levanova and Tanja Westerholm from our collaborative group in Helsinki. Also, the whole population of the 7th floor of Medisiina D and especially the Herpes-group are thanked for a fun and lively year during this Master's thesis research project. Finally, I would like to acknowledge Jane and Aatos Erkko foundation for funding the siRNA swarm -project.

Abbreviations list

10% F-N	An UL29 targeting siRNA swarm, containing 10% of a particular nucleotide (N = A, C or U) 2´-fluoro-modified
100% F-N	An UL29 targeting siRNA swarm, containing 100% of a particular nucleotide (N = A, C or U) 2´-fluoro-modified
88 bp	An 88 bp long, cytotoxic RNA. Used as a positive control for toxicity and innate responses.
ACV	Acyclovir
bp	Base pair
CC_{50}	Half-maximal cytotoxicity
cDNA	Complementary DNA
cGAS	Cyclic GMP-AMP Synthase
CLOCK	Circadian Locomotor Output Cycles Kaput
CMV	Cytomegalovirus
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EC_{50}	Half-maximal efficacy
eGFP	Enhanced green fluorescent protein
E_{max}	Maximum efficacy
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gB	Glycoprotein B (HSV)
gD	Glycoprotein D (HSV)
GFP	Green fluorescent protein
HCE	Human corneal epithelial cell line
HHV	Human herpes virus
HIV-1	Human immunodeficiency virus 1
hpi	Hours post infection
hpt	Hours post transfection

HSE	Herpes simplex encephalitis
HSK	Herpes simplex keratitis
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus 1
HSV-1(17+)	A laboratory reference strain of HSV-1
HSV-2	Herpes simplex virus 2
HSV1-GFP	HSV-1(17+)LoxP$_{mCMV}$GFP, a GFP expressing recombinant HSV-1
HSV1-mCherry	HSV-1(17+)Lox-CheVP26, an mCherry expressing recombinant HSV-1
ICP	Infected Cell Polypeptide, nomenclature for herpes proteins
ICP8	Major DNA-binding protein encoded by UL29 gene of HSV-1
IFI-16	Gamma-interferon-inducible protein
IFN	Interferon-stimulated gene
IgG	Immunoglobulin G
IRF3	Interferon regulatory factor 3
ISG	Interferon Stimulated Gene
LAT	Latency associated transcript
mCMV	Mouse cytomegalovirus promoter
miRNA	Micro RNA
MOA	Mechanism of action
MxA	Myxovirus resistance protein 1
MxB	Myxovirus resistance protein 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nt	Nucleotide
PBS	Phosphate-buffered saline
PET	A non-specific siRNA swarm derived from bacterial lac-operon sequence
pfu	Plaque forming unit(s)
PKR	Protein kinase R
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RSV	Respiratory syncytial virus
RT-qPCR	Quantitative reverse transcription PCR
RT reaction	Reverse transcriptase reaction
siRNA	Small interfering RNA
ssRNA	Single stranded RNA
STING	Stimulator of interferon genes
TLR	Toll-like receptor
U373MG	A neuronal cell line (glioma), reclassified by ATCC as U251
UL	Unique long, a segment of the herpes genome, the genes in the area are named accordingly (e.g. UL29, UL30)
UL29	An essential gene of HSV-1 encoding a protein required for DNA replication in viral replication cycle (ICP8)
UL29.2	A short synthetic siRNA targeting UL29 (Palliser et al., 2006)
US	Unique short, a segment of the herpes genome, the genes in the area are named accordingly (e.g. US3)
VP16	See α-TIF.
α-TIF	Alpha trans inducing factor, initiator of HSV-1 transcription.

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