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INHIBITION OF HERPES SIMPLEX VIRUS INFECTION WITH RNA INTERFERENCE

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To my family!

"…herpes is to say a vocation-…"

P G Unna 1883

ABSTRACT

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Inhibition of herpes simplex virus infection with RNA interference

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Herpes simplex virus (HSV) is a common pathogen. Approximately half of the human population carries the virus. Of clinical symptoms, the most common one is a blister on the lip. This manifestation is often at the initial site of infection. However, in primary infection, before epithelial eradication of HSV by the immune system, the virus infects sensory neurons. In these neurons the virus hides and forms latency. From this latency, the virus can reactivate and travel via axons back to the epithelium to form a new lytic infection. In addition, the virus can upon reactivation travel to the eye and cause HSV keratitis. HSV is the leading cause of blindness due to infectious origin in the developed world. In addition, antiviral resistant HSV strains are relatively abundant in immunocompromised patients and in eye infections of HSV. Thus, there is need for novel drugs against HSV.

In this study, the goal was to develop a new drug against HSV, especially for the treatment of HSV keratitis. RNA interference, based on enzymatically produced and cleaved antiviral small interfering RNA (siRNA) pools (swarms of siRNAs) were studied. The drug development of this therapy started from *in silico* analysis of the target sequences in the viral genome. From there, drugs produced via various methods were studied. Swarms targeting different parts of the viral genome were studied for their innate immunity induction profile and antiviral efficacy. Clinical field isolates of HSV were used in addition to laboratory strains. For an *in vivo* keratitis model, a single siRNA swarm was selected. This swarm, targeting HSV gene *UL29* showed broad effectiveness *in vitro* and *in vivo* and had a limited innate immunity induction profile, being the best candidate for further development.

This study shows, that an siRNA swarm approach against herpes simplex virus infection is feasible.

Keywords: herpes simplex virus, antiviral, RNA interference, innate immunity, drug development

TIIVISTELMÄ

HENRIK PAAVILAINEN
Herpes simplex -virusinfektion esto RNA-parvella

Turun yliopisto, Lääketieteellinen tiedekunta, Virusoppi, Lääketutkimuksen tohtoriohjelma (DRDP)

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Herpes simplex -virus (HSV; yskänrokkovirus) on yleinen ihmisen taudinaiheuttaja. Noin puolet ihmisistä on viruksen kantajia maassamme. Kaikille ei kuitenkaan koskaan tule näkyviä, kliinisiä, oireita. Yleisin oire on huuliherpes. Tällöin virus ja immuunijärjestelmä kilpailevat toisiaan vastaan ja normaalitilanteessa immuunijärjestelmä voittaa. Mutta virus ei ole hävinnyt elimistöltä, vaan se on piiloutunut. HSV infektoi hermosoluja, joiden tumaan virus kulkeutuu ja muodostaa niissä piilevän, latentin infektion. Virus voi aktivoitua uudelleen piilostaan, kulkeutua takaisin epiteelille ja aiheuttaa uuden solutuhoa aiheuttavan infektion. Suun alueen epiteelin sijaan HSV voi kulkeutua myös toisaalle, esimerkiksi silmään ja aiheuttaa HSV-keratiitin. Tällainen silmäinfektio on suhteellisen yleinen ja HSV-keratiitti onkin yleisin infektioperäisen sokeuden aiheuttaja länsimaissa. Hoitoa hankaloittaa se, että nimenomaan HSV-silmäinfektiossa lääkeresistentit viruskannat ovat yleisiä. On siis tarvetta uusille lääkehoidoille.

Tässä tutkimuksessa kehitettiin uutta lääkettä herpesinfektiota vastaan. Erityisenä kysymyksenä oli silmäinfektion hoitoon tähtäävän lääkkeen kehitys. Tutkitut lääkkeet perustuivat RNA-häirinnässä käytettäviin, HSV:n geeneihin kohdistuviin, RNA-parviin. Nämä pieniä häiritseviä RNA molekyylejä sisältävät parvet valmistettiin entsymaattisesti. Lääkekehitysprosessi alkoi sopivien kohteiden seulomisella virusgenomin sekvenssitietoihin perustuen. Eri valmistusmenetelmillä tehtyjä parvia tutkittiin niiden antiviraalisen tehon sekä luonnollisen immuniteetin vaikutusten osalta. *In vitro* -kokeiden perusteella löysimme RNA-parven, joka oli tehokas sekä laboratorio- että kliinisiä HSV-kantoja vastaan. HSV-geeniin *UL29* kohdistuva RNA-parvi aiheutti vain lieviä luonnollisen immuniteetin vasteita ja osoittautui tehokkaaksi myös *in vivo* HSV-keratiittimallissa.

Tämä lääkekehitysprojekti osoittaa, että herpesinfektion estoon suunniteltu RNA-parvi on potentiaalinen lääkekehityssuunta HSV-infektioita vastaan.

Avainsanat: herpes simplex -virus, viruslääke, RNA-häirintä, luonnollinen immuniteetti, lääkekehitys

TABLE OF CONTENTS

ABBREVIATIONS

ACV	acyclovir
BAC	bacterial artificial chromosome
bp	base pair
cDNA	complementary DNA
CMV	cytomegalovirus
CNS	central nervous system
DNA	double stranded deoxyribonucleic acid
dpi	days post infection
EBV	Epstein-Barr virus
EMA	European Medicines Agency
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gC / D / E / I …	glycoprotein C / D / E / I …
GD	*Giardia intestinalis* Dicer
HSCT	Hematopoietic stem cell transplantation
hpi	hours post infection
hpt	hours post transfection
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell protein
IFN	interferon
IgG	immunoglobulin G
ip	intraperitoneally
ISG	interferon stimulated gene
LAT	latency associated transcript
MHC	Major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
NK	natural killer (cell)

nm	nanometer
nt	nucleotide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
qPCR	real-time quantitative PCR
RCF	relative centrifugal force
RISC	RNA-induced silencing complex
(ds)RNA	(double stranded) ribonucleic acid
RNAi	RNA interference
sc	subcutaneously
SEM	standard error of mean
siRNA	small interfering RNA
TG	trigeminal ganglia
tk / TK	thymidine kinase *gene* / protein
TLR3	toll-like receptor 3
U_L	unique sequence long segment (part of HSV genome)
UL27	Unique sequence long segment gene 27
UL29	Unique sequence long segment gene 29
UL54	Unique sequence long segment gene 54
UL27	RNA swarm targeting *UL27*
UL29	RNA swarm targeting *UL29*
UL54	RNA swarm targeting *UL54*
U_S	unique sequence short segment (part of HSV genome)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-IV), and on data presented in this thesis.

I Romanovskaya A*, Paavilainen H*, Nygårdas M, Bamford DH, Hukkanen V and Poranen MM. 2012. E*nzymatically Produced Pools of Canonical and Dicer-Substrate siRNA Molecules Display Comparable Gene Silencing and Antiviral Activities against Herpes Simplex Virus.* PLoS ONE 7(11): e51019. *Equal contribution.

II Paavilainen H, Romanovskaya A, Nygårdas M, Bamford DH, Poranen MM, and Hukkanen V. 2015. *Innate responses to small interfering RNA pools inhibiting herpes simplex virus infection in astrocytoid and epithelial cells.* Innate Immunity 21(4) 349–357.

III Paavilainen H, Lehtinen J, Romanovskaya A, Nygårdas M, Bamford DH, Poranen MM, Hukkanen V. 2016. *Inhibition of clinical pathogenic herpes simplex virus 1 strains with enzymatically created siRNA pools.* Journal of Medical Virology 88(12):2196-2205.

IV Paavilainen H, Lehtinen J, Romanovskaya A, Nygårdas M, Bamford DH, Poranen MM, and Hukkanen V. 2017. *Topical treatment of herpes simplex virus infection with enzymatically created siRNA swarm.* Antiviral Therapy, In press.

1 INTRODUCTION

Herpes simplex virus (HSV) causes diseases in humans. The most common symptom is labial herpes - the most common form of HSV infections of the skin and mucosa. HSV also causes corneal infections and, rarely, HSV encephalitis or meningitis. Approximately every second human is a carrier of HSV, but of them, only a portion gets symptoms. Upon initial infection HSV hides in neurons, causing latency. From this latency, recurrent infection can happen even without symptoms. Antiviral drugs are available against HSV but there is need for improvement, especially as there are drug resistant HSV strains.

Drug development approaches against HSV are broad: from small interfering RNA swarms to monoclonal antibodies to vaccines. The use of small molecule drugs against HSV has not resulted in widespread prevalence of drug resistant HSV strains. However, immunocompromised patients have higher incidence of drug resistant HSV and some current medical treatments result in attenuation of the immune system. Hence, as this type of immunosuppressant treatments are on the increase, it would be likely that more drug resistant HSV cases are going to emerge. Moreover, the drug resistant strains are frequent in HSV keratitis, which is the most common form of HSV eye disease.

RNA interference is a promising approach against infectious diseases. The target of the small interfering (si)RNA can be one or several viral genes. Especially with viruses, who hijack the host cells and rely on the host cell translational machinery for replication, there are less available drug development targets in comparison with bacteria which have a host organism-independent cellular machinery. However, one drawback of single site siRNAs is the risk of drug resistance. A single mutation in the viral genome can render the siRNA ineffective. To circumvent this problem, we have developed enzymatically created siRNA swarms that have a target area of hundreds of nucleotides. These swarms cover a large portion of their target gene. In comparison, the target area is approximately 20 nucleotides for canonical siRNAs.

This thesis focuses on the drug development of an antiviral siRNA swarm against HSV. The long term goal is the treatment of HSV keratitis.

2 REVIEW OF LITERATURE

Viruses are organisms that have a DNA or RNA genome. The can only replicate in living cells using the cellular synthetic machinery to form new particles that have the viral genome. These new particles are then to be transferred to new cells to produce further progeny.

It is easy to answer, whether or not virus is part of the realm of the living; it is. Without life there is no viral activity. But it appears to be more of a philosophical questions whether or not a virus is alive. A virus is fully dependent on its host for replication. Virus is a parasite and is inanimate on its own - the virus particles are not alive outside of the cell

2.1 Herpesviruses

Herpesviruses constitute a family of evolutionary ancient viruses whose origin can be traced back to Pangaean times, when there was only one continent on earth [Grose, 2012]. Members of human herpesviruses, herpes simplex virus (HSV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV), have been found from isolated tribes in South America [Black, 1975], suggesting that these viruses have been there already before the arrival of Europeans. The global distribution of herpesviral genetic clades is in line with the human origins from Africa [Grose, 2012; Hayward, 1999]. For HSV, which is the focus of this thesis, the evolution can be traced for millions of years [Norberg et al., 2011].

Over 200 different herpesviruses belong to the virus family *herpesviridae.* The host range among viruses is wide, with only a few being capable of infecting more than one host organism [Pellett and Roizman, 2013]. Different herpesviruses share the genome type and have a similar virion structure. In the world of viruses, the herpesvirus genome is large, consisting of approximately 152 kb of double stranded DNA. Herpesviruses encode virus-specific enzymes involved in the metabolism of their DNA. Herpesviruses can form lytic and latent infections. In the case of lytic infection, the host cell dies of the infection, with the tendency of producing a high amount of progeny viruses before cell death. As for latent infection, the dormant virus can reactivate to cause a new lytic infection to spread to new hosts. Herpes is a friend for life, as the latent state can last over a lifetime of the host [Forghani et al., 1977], and sometimes even further, as reactivating virus can be isolated from cadavers [Bastian et al., 1972; Ouwendijk et al., 2012].

The herpesviruses are highly adapted to their hosts as both have evolved side by side. Herpesviruses are widely spread, common and rarely cause serious disease in an immunocompetent host. Keeping the host live, well and capable of infecting subsequent hosts is a good survival plan for a virus. However, there are exceptions, and partly due to the sheer magnitude of hosts infected, serious illnesses do occur. The human herpesviruses and the most typical illnesses they cause are presented in **Table 1**.

2.2 Herpes simplex virus (HSV) and us

The first reports of HSV and indeed, the name of the virus, can be traced back to ancient Greeks [Roizman and Whitley, 2001]. Back then, the word "herpes" most likely described many various causative agents of similar disorders. In the 1920's HSV was characterized as an infectious agent in animal studies – in humans, autoinoculation and transmission of HSV was studied and understood already in the 19th century [Flexner and Amoss, 1925; Goodpasture and Teague, 1923a; Roizman and Whitley, 2001; Unna, 1883; Vidal, 1873].

2.2.1 HSV prevalence

Herpes simplex virus is a common human pathogen with approximately half to two thirds of the human population as carriers of the virus [Looker et al., 2015a; Pebody et al., 2004]. However, seroprevalence is decreasing [Bradley et al., 2014]. At any given moment, HSV DNA shedding can be detected from oral scrapings generally in 2-5% of the world population [Roizman et al., 2013]. For example in Finland, a steep drop of HSV seroprevalence from 69.5% to 45% has happened in merely twenty years in pregnant women according to a recent study [Puhakka et al., 2016]. However, in a similar study cohort, over 2% of adults in Finland have HSV DNA positive oral scrapings at a given moment [Mäki et al., 2015]. There are two types of HSV: HSV-1 and HSV-2. HSV-1, which is the main causative agent of oro-labial herpes, and HSV-2, which is considered as the causative agent of genital herpes with an estimated global prevalence of over 11% [Looker et al., 2015b]. HSV took a non-human primate detour 6 million years ago to return as human HSV-2 some 1.6 million years ago whereas HSV-1 has evolved alongside human evolution [Wertheim et al., 2014]. For genital HSV infections, it seems that HSV-1 is becoming more prevalent as the cause of genital herpes in young women [Löwhagen et al., 2000; Roberts et al., 2003; Tuokko et al., 2014].

Table 1. Human herpesviruses.

Common name	Systematic name	Abbreviations	Family[1]	Common clinical manifestations	Global seroposit. %[2]	Discovered[3]
Herpes simplex virus 1	Human herpesvirus 1	HSV-1 HHV-1	α-herpesvirinae →Simplexvirus	Lesion(s), oro-facial area[4]	50-95	[Vidal, 1873]
Herpes simplex virus 2	Human herpesvirus 2	HSV-2 HHV-2	α-herpesvirinae →Simplexvirus	Lesion(s), genitals[5]	5-60	"[Vidal, 1873]"
Varicella-zoster virus	Human herpesvirus 3	VZV HHV-3	α-herpesvirinae →Varicellovirus	Varicella (chicken pox), herpes zoster (shingles)[6]	>95	[Steiner, 1875]
Epstein-Barr virus	Human herpesvirus 4	EBV HHV-4	ɣ-herpesvirinae →Lymphocryptovirus	Mononucleosis, flu like symptoms, Burkitt's lymphoma[7]	>90	[Epstein et al., 1964]
Human Cytomegalovirus	Human herpesvirus 5	HCMV HHV-5	β-herpesvirinae →Cytomegalovirus	Often symptom free / light fever[8] / mononucleosis[9]	52-99	[Lipschütz, 1921]
Human herpesvirus 6A	Human herpesvirus 6A	HHV-6A	β-herpesvirinae →Roseolovirus	CNS symptoms? Roseola[10]	(39-)100[13]	[Salahuddin et al., 1986]
Human herpesvirus 6B	Human herpesvirus 6B	HHV-6B	β-herpesvirinae →Roseolovirus	Roseola infantum, fever[10]	(39-)100[13]	[Yamanishi et al., 1988]
Human herpesvirus 7	Human herpesvirus 7	HHV-7	β-herpesvirinae →Roseolovirus	Fever, seizures, HHV-6 like but older patients[11]	>90	[Frenkel et al., 1990]
Kaposi's sarcoma-associated virus	Human herpesvirus 8	KSHV HHV-8	ɣ-herpesvirinae →Rhadinovirus	Kaposi's Sarcoma[12]	1-60	[Chang et al., 1994]

[1] Herpesvirales→Herpesviridae→ [International Committee on Taxonomy of Viruses, 2016]
[2] [Arvin et al., 2007]
[3] Description of infectivity
[4] [Whitley and Roizman, 2001]
[5] [Gupta et al., 2007; Unna, 1883]
[6] [Cohen et al., 2007]
[7] [Burkitt and O'Conor, 1961; Cohen, 2000]
[8] [Mocarski et al., 2007]
[9] [Klemola and Kääriäinen, 1965]
[10] HHV-6B is the main causative agent of roseola and fever [Dewhurst et al., 1993; Hall et al., 1994]
[11] [Caserta et al., 1998]
[12] [Ganem, 2007; Kaposi, 1872]
[13] HHV-6A and B have not yet been differentiated in large scale epidemiology studies

2.2.2 HSV diseases

As HSV carriage is very common amongst humans, even rarely appearing manifestations can cause a significant medical burden. The most common and arguably the most commonly observed visible symptom is a blister in the oro-facial

area. Even though not necessarily dangerous, a visible blister can be a nuisance both physically and socially. HSV can travel through neurons [Goodpasture and Teague, 1923b] and is capable of forming latency in these neurons (please see below Latency chapter 2.4.2) where it hides from the immune system. From this latency HSV can reactivate and cause a new lytic infection – a lifelong cycle. These reactivations can lead to clinical or subclinical infection, usually near the initial site of infection and can thus transmit the virus to a new susceptible host(s) [Roizman et al., 2013].

However, sometimes this back and forth track is not followed and an alternative site is challenged with a lytic infection. One possibility is that HSV continues onwards to the central nervous system (CNS) where it can cause severe, potentially fatal HSV encephalitis [Steiner, 2011; Whitley and Roizman, 2001]. In encephalitis, lytic viral infection and immune responses both can lead to dire consequences. Moreover, enteric nervous system may be involved as well, as the virus might spread and cause digestive tract damage and peristaltic stop, which has been shown to be the cause of lethality following CNS infection in certain animal models [Khoury-Hanold et al., 2016]. Encephalitis is usually caused by HSV-1 whereas HSV-2 is the more likely causative agent in HSV meningitis. Even though rare, HSV is the most common sporadic cause of encephalitis [Bradshaw and Venkatesan, 2016; Välimaa et al., 2013; Whitley and Roizman, 2001; Whitley et al., 1998].

Another side track HSV can take is the one to the eye. The eye is a somewhat immunoprivileged organ with a constant tear rinsing and a tight protective outer layer. Even though this barrier can be breached, HSV can reach this organ from behind as well, through the nerves [Labetoulle et al., 2000; Labetoulle et al., 2003], as trigeminal ganglia innervate both the eye and the skin of the face [Kuo et al., 2014] (**Figure 1**). In addition to local damage to the eye, HSV infection can result in changes in the trigeminal fibres connected to the area [Rousseau et al., 2015]. The estimated amount of herpes keratitis is in the hundreds of thousands in developed countries (prevalence of approximately 20 out of 100 000 persons/year) and over million in developing countries per annum [Farooq and Shukla, 2012; Labetoulle et al., 2005]. The infection can be persistent and recurrent and HSV is the leading cause of blindness due to infectious origin [Roizman et al., 2013], even with the current medication available in the developed countries.

Hippocrates used the term herpes as lesions that appeared to “creep along the skin” [Roizman and Whitley, 2001] – HSV can cause infections anywhere in the skin or mucosal tissue. A good example of this, still tying to the ancient times, is herpes gladiatorum [Selling and Kibrick, 1964; Wheeler and Cabaniss, 1965]. Of

note, herpes luctatorum (luctator means wrestler in latin) might be historically and linguistically a more appropriate name as gladiators generally wielded weapons and were not as likely to be in such close contact with each other compared to wrestlers [Laur et al., 1979]. Even herpes of wrestlers has been coined as a name [Nomikos et al., 2015]. Nevertheless, herpes gladiatorum remains as the established name for this disease form. The sheer pressure of wrestling can cause microtrauma in the skin, allowing the virus to penetrate the basal layer of cells. From the viewpoint of nurses and dental workers, herpes whitlow (near finger or toe nail) is a medical burden that should be taken seriously [Lewis, 2004; Stern et al., 1959], underlining the need to use gloves when working with herpes.

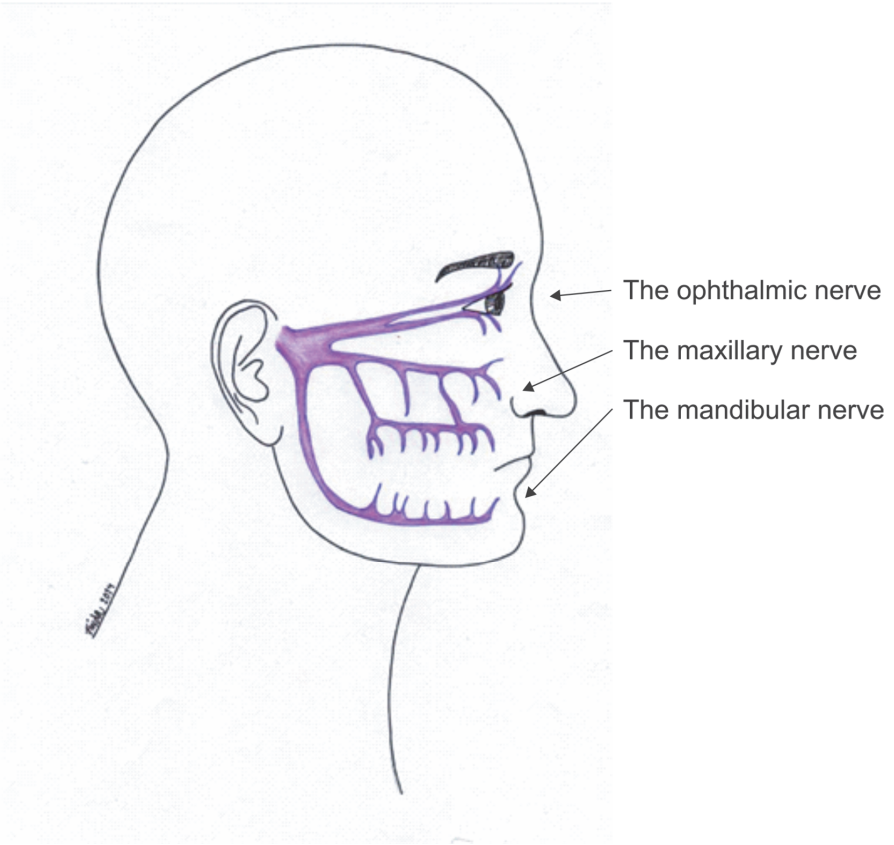

Figure 1. Nervus trigeminus. Trigeminus nerve is divided into three parts; the ophthalmic nerve, the maxillary nerve (upper jaw) and the mandibular nerve (lower jaw). Modified from a picture, courtesy of Anna and Frida Backman, drawn by Frida Backman [Backman, 2014].

HSV spread and infectious capability is not limited to epithelia. A morbid example of this is neonatal herpes, a severe and potentially fatal form of the disease even in the developed countries [Kimberlin, 2004; Nahmias et al., 1967]. This form of the disease is usually contracted during delivery from an ongoing reacti-

vation of the mother's genital herpes and especially in the case of primary infection [Brown et al., 2003]. The infection can become systemic and this can be fatal, even with medication. As the amount of HSV-1 genital infections is on the rise [Tuokko et al., 2014], it is worrying that the systemic infections of the newborn are more likely due to HSV-1 than HSV-2 [Brown et al., 2007; Välimaa et al., 2013].

As an alphaherpesvirus, HSV has a wide variety of host cells. It is in a way an ectodermotopic virus, preferring the epithelium and nervous system, which both originate from the ectoderm. However, HSV can infect many cell types within the body. This facilitates *in vitro* laboratory studies of the virus, where infection and viral replication are desired.

2.2.3 Antiviral medication against HSV

There is currently no herpes simplex vaccine available despite the substantial investments by the pharmaceutical industry. A vaccine candidate in Phase III clinical trial failed recently [Belshe et al., 2012]. This HSV-2 vaccine had some efficacy against HSV-1 genital disease but the efficacy against HSV-2 genital herpes was a disappointment and the vaccine did not reach the market. The scientific community did not stop there, and the amount of HSV vaccine research increased by a third in the following years as measured by amount of publications. There is, fortunately, antiviral medication available against HSV. Acyclovir (ACV), a nucleoside analogue, is a selective drug against HSV [Männistö and Tuominen, 2012; Schaeffer et al., 1978]. To become active, ACV needs HSV gene thymidine kinase (the gene product of UL23/*tk*), and thus the effect is very selective. There are derivatives of ACV, ie. valacyclovir, famciclovir, ganciclovir, which have some effect against other herpesviruses as well. Despite usage around the globe for decades, the resistance prevalence increase is not a highly alarming issue. Prophylactic usage of nucleoside analogues on patients with a risk of contracting the virus has yielded favorable results, but unfortunately not full protection [Anderson et al., 2016]. ACV is a well-tolerated drug, but there is a very low risk that ACV can result in side effects in patients with underlying renal disease; a state called Cotard's syndrome [Lindén and Helldén, 2013]. In addition, prophylactic usage of ACV against HSV cannot entirely prevent viral reactivation [Johnston et al., 2012] and prophylactic usage can promote the emergence of ACV resistant strains [Duan et al., 2009; van Velzen et al., 2013].

As HSV *tk* is a non-essential gene, HSV can replicate without a functional thymidine kinase [Coen and Schaffer, 1980]. A virus negative for *tk* is, however, attenuated, and this can manifest in reduction of spread and replication and reac-

tivation capabilities as well as temperature sensitivity [Coen et al., 1989; Darby et al., 1981; Shimada et al., 2007]. Rarely, HSV DNA polymerase mutation can also lead to ACV resistance in addition to *tk* mutants [Burrel et al., 2013; Chibo et al., 2004; Coen et al., 1985; Larder et al., 1987; Suzutani et al., 2003]. As these strains are often attenuated their detection might be underestimated. ACV resistant HSV usually emerges in immunopriviledged sites such as in the eye, or in immunocompromised patients [Frobert et al., 2014; Stránská et al., 2005]. When an ACV resistant disease emerges, the drug options are few. Foscarnet is available [Männistö and Tuominen, 2012], but the drug is not well tolerated, and over half of the treated patient's HSVs generate resistance to it [Danve-Szatanek et al., 2004]. Moreover, TK can remain (somewhat) functional but at the same time drug resistant [Darby et al., 1981]. Even with all the available antivirals, HSV remains a significant medical burden and there is a need for treatment modalities with differing mechanisms of action.

2.3 The structure of herpes simplex virus

2.3.1 HSV virion

A description of an HSV virion in an electron micrograph is a sunny side up fried egg (**Figure 2**). The 3D shape is roughly a sphere, but a flexible one. The size of a virion is 225 nanometers (nm) with glycoprotein spikes protruding from the lipid envelope and 186 nm without [Grünewald et al., 2003].

The structure is from center outwards: the genome, capsid, tegument, and lipid envelope (**Figure 2**). The outer layer of the virion is the lipid envelope harboring viral glycoproteins. Underneath lays the tegument. Its structure is not rigid and it harbors numerous HSV proteins, including virion host shutoff protein, which upon release to the host cells starts to degrade host messenger RNAs (mRNAs) [Kwong et al., 1988; Read and Frenkel, 1983]. HSV capsid is an icosahedral protein structure [Schrag et al., 1989]. Its size is one-third of the lipid enveloped volume [Grünewald et al., 2003]. The capsid shelters the viral DNA, which is released to the nucleus of the cell once the capsid has been transported there via microtubules [Marozin et al., 2004; Ojala et al., 2000]. Viral DNA is held in the core of the virion inside the capsid. Here the DNA is held in a toroid form [Furlong et al., 1972; Kieff et al., 1971] and the negative charge of the DNA is balanced by the presence of polyamins, spermidine and spermine [Gibson and Roizman, 1971]. The DNA is in linear form within the capsid, but upon entry to nucleus, the DNA takes a circular episomal form and associates with histones [Garber et al., 1993; Kent et al., 2004].

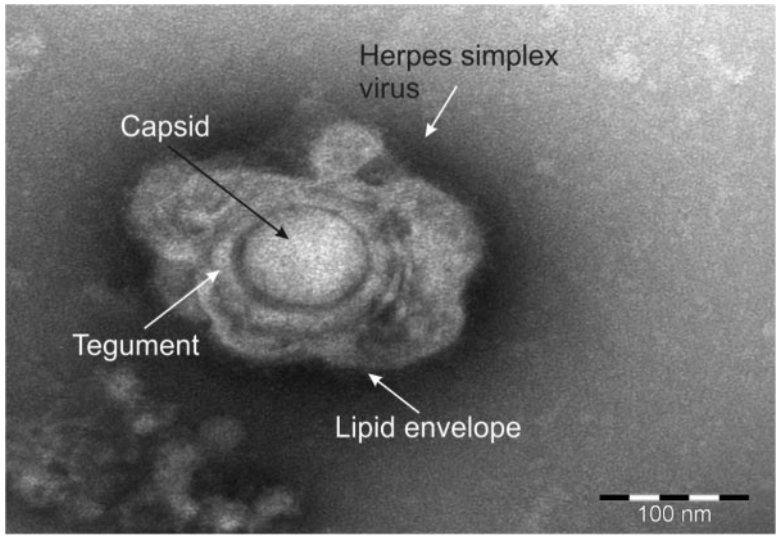

Figure 2. Herpes simplex virus virion. An electron microscopy picture of HSV. Courtesy of Outi Heikkilä.

2.3.2 Genome of HSV and its role in drug development

Herpes simplex virus has a double stranded DNA genome (**Figure 3**). The first sequenced HSV genome was that of HSV-1 strain 17+, which has a genome length of 152261 base pairs (bp) [Dolan et al., 1998; McGeoch et al., 1988] [GenBank: X14112.1]. Strain 17+ is also reported of being 152222 bp long [NCBI Reference Sequence: NC_001806.2]. HSV has a very high GC content (67% for HSV-1 and >69% for HSV-2 [Dolan et al., 1998; Kieff et al., 1971]), large invert repeat areas, and small but numerous tandem repeats. Thus far, many HSV-1 and -2 strains have been sequenced and the lengths vary but not drastically. However, even with next generation sequencing, certain areas of the HSV genome have been proven to be notoriously difficult to resolve efficiently. To make things even more complicated, a virus strain, such as 17+, seems to be a mixture of slightly different viruses with varying genomes, rather than a unison of a single genome [Parsons et al., 2015]. Moreover, partial drug resistance of a clinical isolate strain can be detected, or at least within the infected patient [Biswas et al., 2007; Sukla et al., 2010]. In other words, resistance to a drug candidate with novel mechanism can pre-exist in a single viral isolate [Biswas et al., 2007]. However, pre-existing resistance to a candidate drug does not automatically lead to a cease in drug development [Katsumata et al., 2012; Maruho Co Ltd, 2016; Tyring et al., 2012].

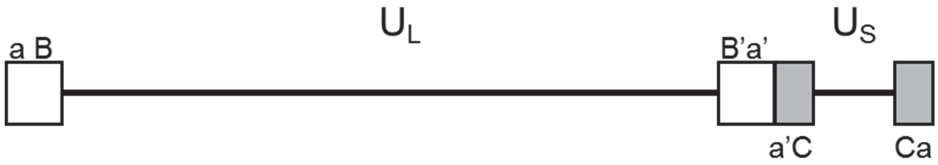

Figure 3. Schematic representation of the HSV genome. U_L = unique sequence long segment, U_S = unique sequence short segment, B/B' = repeat and its inversion flanking U_L, C/C' repeat and its inversion flanking U_S, a/a' = short few hundred base pair repeat.

Thus far, at least 90 transcriptional units have been found from HSV genome and of these at least 84 encode proteins (www.viprbrc.org/). The genes are transcribed in opposite directions and can be overlapping. Latency associated transcript, LAT, a long non-coding RNA with an initial length of approximately 9 kb and with the subsequent shorter stable 1.5 and 2.0 kb LAT introns and micro (mi)RNAs all have a role in maintenance of HSV latency (and please see 2.4.2) [Cliffe et al., 2009; Stevens et al., 1987; Umbach et al., 2008]. HSV miRNAs are expressed during lytic infection cycle as well [Jurak et al., 2010]. No known proteins are expressed from LAT region in latency, even though there are a handful of short open reading frames that can be expressed during lytic infection [Lagunoff and Roizman, 1994].

In a lytic infection, HSV genes are expressed in a cascade fashion with α genes first, followed by β and then ɣ genes [Honess and Roizman, 1974]. In a latent infection, these genes are silenced. Upon reactivation the gene expression is not regulated in expression cascade at first as all gene classes are expressed simultaneously and then followed by a more classical expression cascade [Camarena et al., 2010; Du et al., 2011; Mattila et al., 2015]. Some of HSV genes are not important/necessary for its replication in cells *in vitro*, however, a loss of gene function usually results in attenuation in virulence in a host organism. Noteworthy is the ɣ34.5 gene known as the neurovirulence gene [Bolovan et al., 1994; Chou et al., 1990], coding for multifunctionary infected cell protein (ICP)34.5 [Ackermann et al., 1986; Alexander et al., 2007; He et al., 1997], as its deletion leads to a virus that does not harm the CNS but can still selectively (ie. cancer) be a lytic virus [Andreansky et al., 1998; Andtbacka et al., 2015; Broberg et al., 2001; Markert et al., 2000; Nygårdas et al., 2013].

2.4 Infection cycle

There are two major phases in herpes simplex virus life cycle; the lytic and latent infection. In lytic infection, the virus replicates and produces new virions with the result of lysis of the infected cell. Hallmark of viral spreading *in vitro* is plaque formation (**Figure 4**), based on lateral spread of the virus with the help of viral glycoproteins [Dingwell et al., 1994].

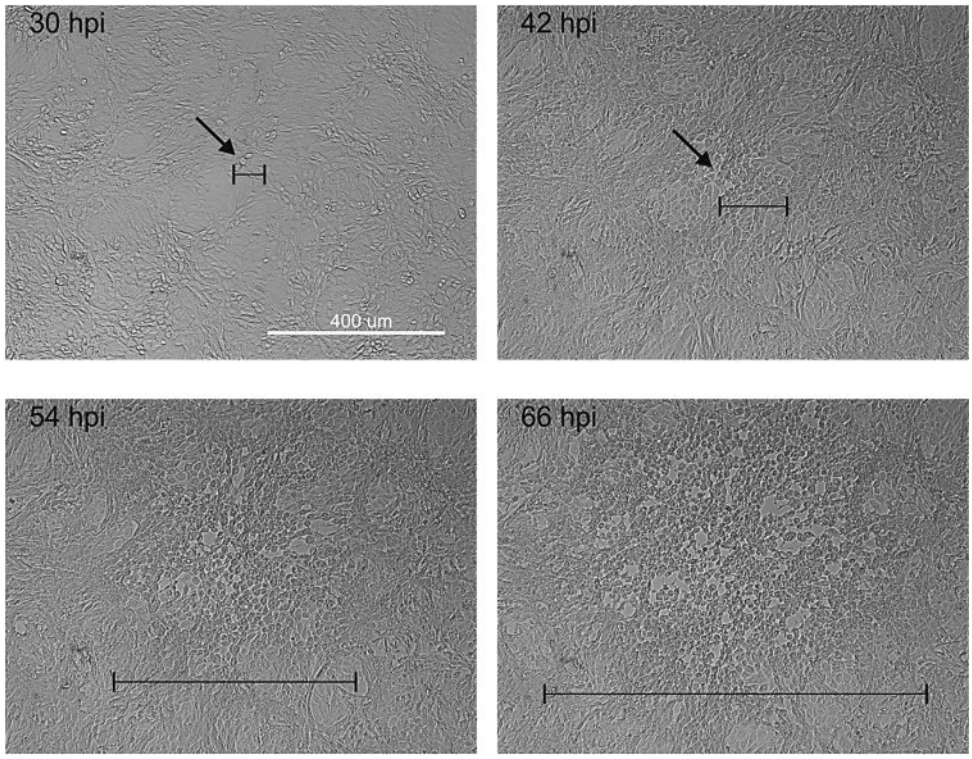

Figure 4. HSV plaque formation. The developing HSV-1 plaque is indicated by an arrow in the panels representing 30 and 42 hours post infection (hpi). The black bar depicts the width of the plaque. All pictures are to scale and the scale bar is shown in the 30 hpi panel. Vero cells were infected at a low MOI and the developing HSV-1 plaque was analyzed at 30, 42, 54 and 66 hpi.

An *in vivo* clinical example of the spread is a blister. When this infection is ongoing, the virus is detectable by the immune system (please see section 2.5). Thus, if an immune system is present and active, the spread at lytic infection site is halted. The relatively quick inhibition of the viral spread is an important safety feature for the virus, as it is advantageous for the virus to keep the host alive and capable of infecting new individuals. To start a new virus production cycle, there are two possibilities, infect a new host or hide from the immune system. HSV is a master of stealth, and in latent infection the virus cannot be eradicated by the

immune system and from this quiescent form, the virus can reactivate to cause a new lytic infection.

2.4.1 Lytic infection

The chain of events triggered by lytic infection is the reason for symptoms, which occur during HSV infection. Thus, this phase of the infection is a natural target for drug design, but there are, however, some approaches trying to eradicate latent virus from the host as well (as pointed out in **Table 2**, 2.8). The virus enters the cell through fusion of lipid envelopes of HSV and the host cell [Morgan et al., 1968]. An alternative pathway involving endocytosis exists as well [Nicola et al., 2003]. HSV glycoproteins play an indispensable role in entry pathways and this complex and variable setting, with both the virus and the host cells playing their parts, is still unfolding, as reviewed by [Campadelli-Fiume et al., 2012; Heldwein and Krummenacher, 2008]. Upon entry, the contents of the viral tegument are released into the cell. Elements from the tegument are accompanied by HSV infection-related exosome-delivered factors [Kalamvoki et al., 2014]. These factors have an effect on the antiviral responses by the cell and on the expression of the viral genes later on in the infection. The viral DNA, encapsulated in the capsid, is transported to the nucleus by microtubules [Kristensson et al., 1986] with dynein where the linear DNA is released [Döhner et al., 2002; Ojala et al., 2000; Sodeik et al., 1997]. The DNA takes a circular form [Strang and Stow, 2005; Su et al., 2002], and provided that the host cell and viral factors are suitably present, starts to express viral genes, resulting in a complete takeover of the cell for viral production.

As mentioned above, the viral genes are expressed in a cascade. In a simplification, α gene expression is required for β gene expression and β gene expression is required for ɣ gene expression. Already two hours post infection (hpi), α genes are expressed, whereas β gene expression begins 4 to 8 hpi. After viral DNA synthesis has commenced, the ɣ genes start to be expressed. Finally all the parts of a new virion are produced, and the egress of new infective viruses can commence. When infection is elaborated more closely, HSV genes are “leaky” for the different gene classes [Powell et al., 1975]. In addition, the α genes are defined by their ability to be expressed in the presence of cycloheximide (ie. without de novo protein synthesis), and the β genes are not expressed prior to α gene expression after cycloheximide removal [Honess and Roizman, 1974]. In the presence of phosphonoacetic acid, α and β genes are readily expressed whereas ɣ genes are not [Honess and Watson, 1977]. Moreover, reactivation from a latent infection to productive infection is proceeded with non-cascade type of gene expression [Camarena et al., 2010; Mattila et al., 2015].

2.4.2 Latent infection

In latent infection, the virus hides from its host. Upon infecting latently a new host cell, the viral genome is repressed. This repression could also be viewed as a natural state of the virus. Two neuronal populations are found upon acute infection; one in which viral genes are expressed and another, where lytic infection associated-genes are repressed [Margolis et al., 1992]. While in hiding, only a part of the viral genome is active, the latency associated RNA, LAT, is expressed [Stevens et al., 1987; Wagner et al., 1988]. In addition to LAT and its splice variants, also miRNA are expressed from the latency region of the virus [Jurak et al., 2010; Umbach et al., 2008]. The viral DNA stays in the nucleus as a non-integrated episome. The DNA is bound to histones with the LAT area to acetylated [Kubat et al., 2004] and lytic genes to dimethylated histones [Wang et al., 2005]. This results in the activity of the LAT area and the inactivity of the lytic genes. Latency forms in sensory neurons in which the capsid is transported via retrograde transport into the soma of the neuron, where the viral DNA stays (non-integrated) in a latent state in the nucleus. The requirement for definition of genuine latency is that the virus can reactivate from this state. Upon reactivation the repressing locks open and the anterograde transport takes the infection to, or near to, the original infection site. There can be deviations from this, as in rare cases the virus travels the wrong way to the CNS. In addition, infection can manifest at another peripheral site than the initial site of infection – for example from a primary lip infection, a recurrent infection in the eye can ensue upon viral reactivation.

The complexity of the latency-reactivation cycle and equilibrium of immune system and the virus is baffling. A good example of the equilibrium is that an adapted T cell population is waiting for HSV to reactivate at the previous site(s) of infection *in vivo* [Gebhardt et al., 2009]. Despite this alertness, HSV can form a new lytic cycle.

Understanding latency is a daunting task, but could indeed open up new possible targets for treatment of HSV infection. However, for example, cutting the (latent) genome with various methods has not thus far led to desired results [van Diemen et al., 2016], but not without some preliminary success as well [Aubert et al., 2014].

2.5 HSV and the immune system

Immune system is crucial for human survival. It consists of many barriers that a pathogen must overcome. Parts of it we are born with (the innate immunity), and

parts adapt to pathogens (the adaptive immunity). These two parts of immunity are integrated to one another. The first barriers of the immune system are physical, such as the skin and the low pH of the stomach. On a tissue level, the immune cells can autonomously detect pathogens and, additionally, adapt to them. Both pathogens and immune system adapt to counteract the other's countermeasures. On a subcellular level, antibodies and the complement system play an integral part in the detection and destruction of pathogens. Some cells are solely dedicated to immune responses, but cells in general have a possibility to react to pathogens. Interferons help the (infected) cell to counteract viral infections, alert neighboring cells and call for help from other cell types.

Many immune system processes such as complement system, natural killer (NK) cells and T- and B-cell responses all try to eradicate HSV. Functioning immune system can clear the lytic infection and thus it will not spread too much in the host. However, latent HSV remains even with the efforts of a functional immune system.

Even though immune evasion is important for the virus survival, in a way HSV relies on many innate immunity systems to slow it down enough not to cause too much havoc on the host. Even viruses where the neurovirulence gene has been deleted remain virulent in nude mice, with partly deficient immune system [Lasner et al., 1998]. Toll-like receptor 3 (TLR3), part of the innate immunity pathogen pattern associated recognition, for one, is important in the HSV encephalitis prevention [Zhang et al., 2007]. NK cells can locate the viruses so that adaptive immunity can step in line, but not before the virus has had an opportunity in the production of new virions. Immunocompetent hosts rarely get serious infections.

2.5.1 Innate immunity against HSV

The first and perhaps the best barrier against HSV infection is the intact skin. Other factors, such saliva, also have antiviral effect on HSV [Välimaa et al., 2002]. To replicate, HSV needs access to the basal layer cells. Upon infection (entry to the host cell), the host cell responses are shut off. Host gene expression is inhibited, cellular proteins are degraded and programmed cell death is blocked, to a point. Complement alternative (and classical) pathway activation is inhibited by HSV glycoprotein C (gC) blocking C3b component of complement system [Fries et al., 1986; Kostavasili et al., 1997]. On the other hand, however, antibodies can target gC [Adamiak et al., 2010]. Major histocompatibility complex (MHC) class I presentation of virus is inhibited to hide from T-cell mediated responses (see below) but this inhibition leads to susceptibility to NK cell respons-

es. NK response is not that effectively inhibited by HSV. However, (at least) HSV-2 has the ability to deceive NK cells' function with secreted glycoprotein G-2 [Bellner et al., 2005]. The importance of innate immunity against HSV is underlined by the fact that TLR3 deficiency subjects children to encephalitis [Zhang et al., 2007].

2.5.2 Adaptive immunity responses to HSV

Adaptive immunity is blocked in multiple ways by HSV. Antigen presentation is inhibited by ICP47. ICP47 prevents peptide loading to MHC and prevents their transport for CD8+ T cell presentation [Früh et al., 1995; Hill et al., 1995; York et al., 1994]. HSV can prevent T cell mediated cell death by other ways as well, but these mechanisms are not as clearly elucidated. Antibody-mediated adaptive immunity is blocked by gE and gI that form a Fc receptor that, through binding to Fc part of immunoglobulin G (IgG), blocks antibody-dependent antiviral responses [Johnson et al., 1988]. In addition, gC and gE block HSV recognition via antibodies by sheltering other viral glycoproteins [Hook et al., 2008]. An indirect evidence of HSV's capability to evade adaptive immunity is the fact that there is no vaccine available. This is the case despite the investments in and devotion to creating one by the industry and academic research.

2.6 HSV infection models

It has been nearly hundred years since the beginning of experimental HSV infection models as reviewed by [Roizman and Whitley, 2001]. Even though HSV is a human virus, it can, through inoculation, infect various animals and animal cells from mouse to monkey through zebrafish to chicken, to name a few [Burgos et al., 2008; Flexner and Amoss, 1925; Hukkanen et al., 2002; Hunter et al., 1999; Scott et al., 1953; Wertheim et al., 2014]. A wide range of hosts offer different models in which the virus can be studied. On the other hand, this creates a necessity of choosing the most suitable model for each experimental need.

2.6.1 In vitro infection

An *in vitro* setting is the method of choice for viral production. For HSV, many factors such as temperature can have an effect on viral production [Hoggan and Roizman, 1959] and spread [Hoggan et al., 1960], different cells produce different amounts of virus [Kaplan, 1957] and different viral stocks have different

spread and replication properties [Roizman and Roane, 1961]. Moreover, cell lines can lack the capability to produce interferons, and for that matter be more suitable for viral (stock) production [Desmyter et al., 1968]. More recently, advanced experimental settings can model more complex infections than lytic infections, such as latency [Camarena et al., 2010; Hafezi et al., 2012; Mattila et al., 2015; Wilcox and Johnson, 1987]. Thus the knowledge and/or the exploration, of the *in vitro* system used, is very important.

In the context of drug development, a good lead in *in vitro* phase is important. There is not, however, a need for hundred percent inhibition of viral replication to be a promising lead. In addition, broad spectrum antiviral molecule might have cell type specific activity [Denisova et al., 2012]. As discussed above, acyclovir is a good drug against HSV. Nevertheless, its *in vitro* efficacy in viral inhibition (decrease in viral replication) was 2-4 logs compared to untreated cells [Rosenwirth et al., 1987] and combination treatment with interferon (IFN)-α increased the inhibition of viral replication by ACV from 2 to over 3 logs [Taylor et al., 1989]. This seeming discrepancy with these results merely demonstrates the difference between experimental settings and the subsequent findings, not the reduced activity of the drug. Another factor, which needs to be taken into account, is the possible interaction of a drug with cellular defenses, such as apoptosis, which can lead to destruction of infected cells. If a drug is working in such a way, but it is intended against cancerous cells, not infected cells, the results can be potentially very harmful for the host as cell destruction can happen at an undesired location [Kakkola et al., 2013].

2.6.2 In vivo infection models

In comparison with *in vitro* models, *in vivo* models face even more complications; each factor contributes to overall pathogenesis of the virus. The strain and production method of the virus, as well as the strain, age and sex of the animal are just a few factors contributing to the infection course [Lopez, 1975]. For example the Swiss Jim Lambert (SJL) mice are more susceptible to latent infection through the intranasal infection route rather than the more commonly used ocular route [Nygårdas, 2013]. In addition to animal strain, also the age of the animal has an effect on the susceptibility to HSV as older mice can tolerate much higher viral doses than younger ones as the pups do not develop an NK cell response [Zawatzky et al., 1982]. Mouse is perhaps the most used animal for HSV infection. Major drawbacks of the mouse as a model animal for HSV infection is the lack of (detectable) spontaneous reactivation and that the HSV immunoevasion factor ICP47 is not active in mice. Reactivation can be induced for example with

another infection [Stevens et al., 1975], heat shock [Sawtell and Thompson, 1992] and with severe organ stress, such as death of host followed by explant culture of ganglia [Baringer, 1976; Stevens and Cook, 1971]. Mouse does, however, offer an extensively studied and robust setting for HSV infections. Corneal HSV infections are studied mostly in rabbits, or C57BL/6 and BALB/c mice [Hill et al., 2012; Nygårdas, 2013]. In comparison to mouse models, spontaneous reactivation happens in rabbits after corneal infection and in guinea pigs after genital inoculation [Wagner and Bloom, 1997]. For genital herpes models, guinea pig is often used [Da Costa et al., 1997], but mouse [Palliser et al., 2006] and rat models are used as well [Boukhvalova et al., 2015; Yim et al., 2005]. Non-human primates are used as well, albeit more rarely, for HSV related studies, but they do, however, have their role in stepping from pre-clinical to clinical research and different animal models are used to meet and represent various experimental settings [Meignier et al., 1987; Meignier et al., 1988; Meignier et al., 1990; Patel et al., 2016; Roth et al., 2014]. Newest member of host species in HSV infection studies *in vivo* is the tree shrew, with the interesting distinction of ICP0 transcript expression during latency [Li et al., 2016]. Should this or similar expression be true in humans as well, there might be great deal of impact on antiviral development against latent HSV. For lytic HSV infections, however, the current models offer a good and wide selection for drug development.

2.7 HSV as a tool

2.7.1 Gene therapy applications of HSV and their impact on antiviral drug development

In the gene therapy and virotherapy field, HSV has played a key role. Most notably, replication competent HSV was “the first FDA-approved oncolytic virus therapy” [FDA, 2015] (U.S. Food and Drug Administration, FDA) and European Medicines Agency (EMA): “Imlygic is a first-in-class advanced therapy medicinal product (ATMP) derived from a virus” [EMA, 2015]. Deliberate attenuation of HSV, and addition of therapy genes, has led to first oncolytic virus drug on the western market [Andtbacka et al., 2015; Duodecim-drug-database, 2016; Hukkanen and Vihinen, 2016]. In addition to cancer, replication competent HSV vectors are in development against autoimmune disease as well [Broberg et al., 2001; Nygårdas et al., 2011; Nygårdas et al., 2013].

Manipulation of HSV genome has been done for over 30 years [Post et al., 1981]. The original tandem transfection method is still used, with the bacterial artificial

chromosome (BAC) *en passant* technique as a current alternative [Brunnemann et al., 2016; Nagel, 2006; Nygårdas et al., 2013; Tischer et al., 2006]. With the BAC technique, there is no need to work with a live virus while modifying the genome. The genome modifications are based on homologous recombination. HSV genome (**Figure 3**) with its repeat areas forms homologous recombination within itself in a eukaryotic cell. In the gene therapy drug development field, this natural and laboratory-induced recombination can be exploited and the methods designed so that there is virtually no risk of increasing virulence mutations. In addition, the natural drug sensitivity gene is present. However, the modifications to the *tk* gene in the gene therapy applications would be in some cases desirable [Manservigi et al., 2010; Wilson et al., 1999]. Due to (current) lack of other suitable drugs, the drug sensitivity gene deletion is not a favorable choice in vector development. In addition, a situation where one needs to medicate against HSV and let HSV-based therapy vector function at the same time is quite likely faced in the future. Therefore, there is a need for HSV drugs with varying methods of action.

Parts of HSV have been exploited for other gene therapy applications, namely the *tk* gene. An obvious choice has been the incorporation of the *tk* gene to a heterologous oncolytic viral vector and, once the tumor cells are infected, a *tk*-based drug is given to the patient with the result of the infected cells dying [Sangro et al., 2010; Stedt et al., 2013; Su et al., 1996]. This short term exposure to *tk*-based drug is unlikely to significantly influence serious emergence of drug-resistant herpes strains [Mitterreiter et al., 2016].

Another gene therapy approach, with more relevance to anti-HSV drug development, is the use of *tk*-positive lymphocytes in (haplo-identical hematopoietic) stem-cell transplantation (HSCT). The idea is to reserve a safety switch for graft-versus-host disease as the given modified-to-be-*tk*-positive-cells can be destroyed with drug (ie. ganciclovir) treatment in the case of adverse effects [Ciceri et al., 2009; Hashimoto et al., 2015a]. Unfortunately, these patients are susceptible for herpes infections; giving the *tk*-based drug against manifestation of HSV would also trigger the off-switch of the HSCT treatment. Moreover, HSCT patients are at greatest risk of *tk*-resistant HSV prevalence [Piret and Boivin, 2011]. This leads to the problematic phase where treatment of viral infection might be unsuccessful due to resistance but at the same time the safety switch is triggered unnecessarily. In addition, *tk*-positive lymphocyte-treated patients that need to be treated with *tk*-based drug, can alter the remaining *tk*-positive lymphocytes [Hashimoto et al., 2015b]. Furthermore, *tk*-based drug prophylaxis is considered a standard procedure for herpes(simplex virus) seropositive individuals [Tomblyn et al., 2009], and this again can result in the emergence of new resistant viruses [Duan et al., 2009; van Velzen et al., 2013].

All in all, there are numerous reasons closely knitted to current and future gene therapy applications, why drugs with new mechanisms of action are needed against HSV.

2.8 Drug development against HSV

As a virus that has spread ubiquitously around the globe in the human population, HSV has remained as an attractive target for drug development (**Table 2**). Big pharma, amongst others, has put a lot of effort in HSV vaccine development. An HSV-2 vaccine [Belshe et al., 2012], was found to be slightly active against HSV-1 in its phase III trial. Alas, the result was disappointing as it did not have an effect on HSV-2 genital herpes. Genital herpes infection control is important, since in addition to HSV medical burden, genital HSV infection increases the risk of contraction of HIV [Freeman et al., 2006; Wald and Link, 2002].

As discussed previously, there is a good drug against HSV, acyclovir and its derivatives, which all base their effect on the *tk*-gene. However, *tk*, being an important yet dispensable gene, allows for escape mutants to emerge [Duan et al., 2009; Piret and Boivin, 2011]. Even slightly active thymidine kinase allows for reactivation of the virus [Pan and Coen, 2012] and thus a new recurrent infection to which new drugs are needed. There are various approaches to combat HSV infection and a set of these approaches is listed in **Table 2**.

2.9 RNA interference

RNA interference (RNAi) is a built-in mechanism of gene silencing in eukaryotes [Cerutti and Casas-Mollano, 2006]. MicroRNAs use this pathway in normal cellular function. Viruses, including HSV, use miRNAs as well [Jurak et al., 2010; Umbach et al., 2008]. The key component is a short double-stranded (ds)RNA strand that is used to detect and lead to destruction of the messenger RNA (**Figure 5**).

Table 2. Examples of drug development approaches against herpes simplex virus.

Targeted virus	Mechanism	Targeted disease phase	Research status	Reference
HSV	Enzymatically created siRNA swarm	Lytic	In vivo	This thesis
HSV-1	Chemically synthesized siRNA	Lytic	In vivo	[da Silva et al., 2016; Li et al., 2014; Palliser et al., 2006]
HSV-1	Monoclonal antibody	Lytic	In vivo	[Krawczyk et al., 2015]
HSV-1	Homing megaendonuclease	Latent (lytic)	In vitro (in vivo?)	[Aubert et al., 2014; Grosse et al., 2011]
HSV-1	Adenovirus mediated shRNA	Lytic	In vitro	[Song et al., 2016]
HSV	Crispr/CAS9	Lytic	In vitro	[van Diemen et al., 2016]
HSV	Small molecule (microbicide)	Lytic / prophylaxis	In vitro	[Chamoun-Emanuelli et al., 2014]
HSV-2	HSV-1 (amplicon) shRNA vector	Lytic, recurrence	In vivo	[Liu et al., 2013]
HSV-2	Replication defective HSV-2	Prophylactic, lytic, latent – vaccine	Phase I	[Bernard et al., 2015; Da Costa et al., 2000]
HSV-2	Attenuated HSV-1 (ICP0 mutant)	Vaccine	In vivo / Phase 0?	[Halford et al., 2010]
HSV-2	DNA	Vaccine	Phase I	[Dutton et al., 2013]
HSV-2 (-1)	Subunit (gD)	Vaccine	Phase III, discontinued	[Belshe et al., 2012]
HSV-2	ΔgD HSV-2	Vaccine	In vivo	[Petro et al., 2016]

To create these short RNAs, commonly referred to as small interfering RNAs (siRNA), miRNAs are loaded into Dicer. Dicer is an enzyme that cleaves the long dsRNA to siRNA [Bernstein et al., 2001]. Length of the siRNA depends on the Dicer. Human Dicer cleaves 21-23 bp long siRNAs [Provost et al., 2002], while for example *Giardia intestinalis* Dicer cleaves 25-27 bp long siRNAs [Macrae et al., 2006]. These longer siRNAs are considered Dicer-substrate siRNAs, as they are loaded to (human) Dicer upon entering cells of human origin. After siRNA is available, it enters the RNA-induced silencing complex (RISC) [Hammond et al., 2000]. There the two strands are separated and the guide strand is associated with Argonaute 2 protein. The Argonaute 2 mediates sequence-specific cleavage of the complementary mRNA [Rand et al., 2004].

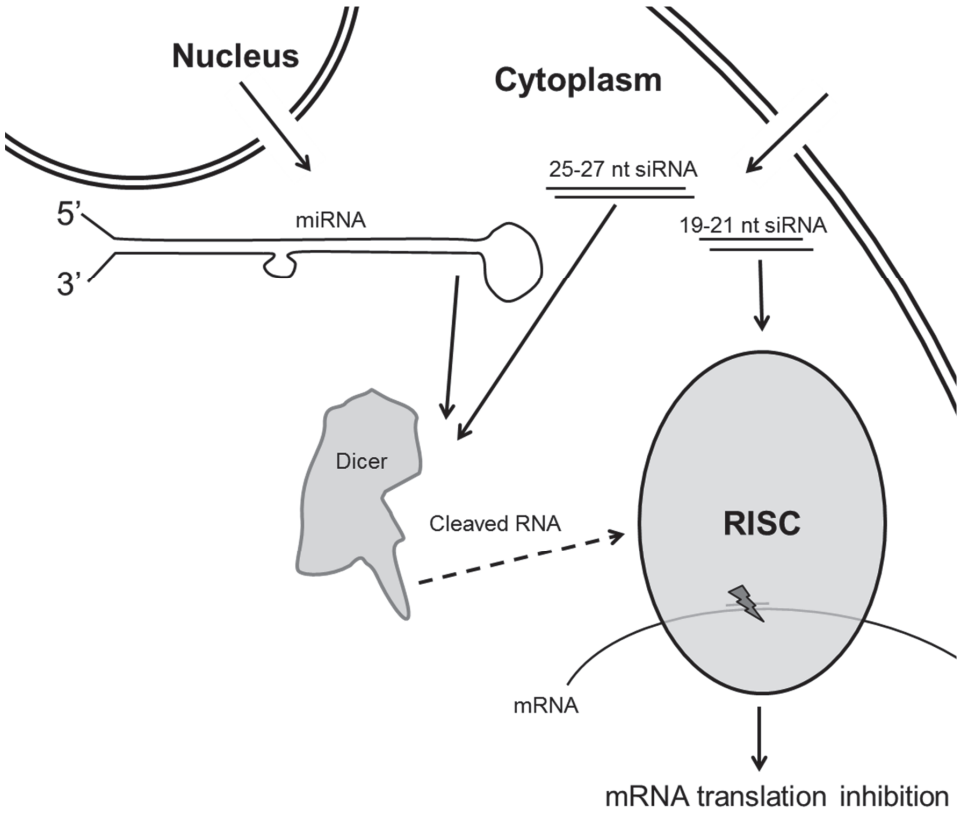

Figure 5. **RNA-interference in a human cell.** Endogenous or exogenous RNA molecules are cleaved by Dicer (Dicer-substrate RNA) and then loaded to RISC complex via which sequence specifically mRNA translation is inhibited. Short siRNA molecules can bypass Dicer loading. RISC = RNA-induced silencing complex, siRNA = short interfering RNA, miRNA = microRNA, mRNA = messenger RNA.

2.9.1 RNA interference in drug development

Exogenous siRNAs can trigger the RNAi pathway as well [Elbashir et al., 2001]. There are multiple approaches in drug development field to combat various diseases with an siRNA approach, ranging from viral infections to cancers to age-related macular degeneration to name a few from currently ongoing or finished clinical trials [ClinicalTrials.gov, 2016]. In preclinical phase the choices are even more numerous.

As a biological drug, the manufacturing of siRNAs is relatively simple. Chemical synthesis can be used of a chosen sequence and such molecules can be ordered from commercial vendors. Another approach is to enzymatically create a swarm

of siRNAs, siRNA pools [Aalto et al., 2007; Donzé and Picard, 2002; Myers et al., 2003; Nygårdas et al., 2009; Romanovskaya et al., 2013; Yang et al., 2002]. A part of a gene is chosen as a template and from this, hundreds of base pairs long target area, a long dsRNA molecule is synthesized. Depending on Dicer used to cleave siRNAs from the long dsRNA, the final swarm of siRNAs can be of varying lengths. When aiming to treat viral diseases with siRNAs, especially with siRNA products targeting single site of the target gene, the risk of emergence of resistance is a problem [Geisbert et al., 2006; Gitlin et al., 2005; McDonagh et al., 2015; Shah et al., 2012; Wilson and Richardson, 2005]. When the target area covers hundreds of base pairs, the risk of resistance mutants is unlikely to occur quickly. In the pools, the amount of single siRNA molecules with identical sequence is low, and thus off-target effects due to siRNA sequence are very low. Unintended effects due to exogenous RNA can be also due to the introduction of foreign dsRNA into the cell. The length of the dsRNA is an important factor in the response, and, to point, generally the longer the dsRNA is, the stronger the toxic effect is. For example, 88 bp long dsRNA is considered toxic, and even more toxic than hundreds to over thousand bp long dsRNAs [Jiang et al., 2011]. It induces lethal cellular responses, by being detected by cellular sensors of dsRNA. For shorter siRNAs (<30 bp), the increase of size does not necessarily lead to intensifying cellular responses. In some cases, for chemically synthesized siRNAs, the increase in length of the siRNA adds to toxicity [Reynolds et al., 2006]. However, the sequence itself can have an effect on the non-target specific responses [Fedorov et al., 2006]. In human cells, the difference between a shorter and longer siRNA is the route to RISC complex; longer siRNA first goes through Dicer before being loaded to RISC whereas shorter siRNAs are directly loaded to RISC [Kim et al., 2005] (**Figure 5**). This factor could contribute to the different induction of innate immunity in various size RNA swarms.

The delivery of siRNA drugs is often the main question in clinical development, as the target tissue can be hard to reach. The siRNAs are also somewhat delicate and large molecules, compared to canonical small molecule drugs, and thus *per os* administration is unlikely to render good bioavailability for siRNAs. Many approaches are under development to help with the homing of the RNAi based drugs including protein and other linker conjugations, lipid envelopment, delivery vectors such as viruses, transfection, nanoparticles, and of course plain naked siRNA [da Silva et al., 2016; Kanasty et al., 2013; Kari et al., 2007; Kim et al., 2016]. No blockbuster RNAi based drugs have made it to the market, but there has been an antiviral antisense oligonucleotide drug approved by the FDA [Crooke, 1998], paving the way for new RNAi biologicals in the future.

2.9.2 Use of RNAi against herpes simplex virus

There are a few efforts pursuing to concept of HSV infection inhibition via chemically synthesized small interfering RNAs [Duan et al., 2012; Jin et al., 2014; Palliser et al., 2006; Silva et al., 2014]. RNA interference is a promising antiviral approach against HSV. The mechanism of action differs clearly from current available treatments. Most common manifestation of the disease is at the periphery. When planning the delivery of siRNA, a biological drug with a relatively large molecular mass compared to small molecule drugs, skin, eye, mucosal membranes are all sites that are easier to reach than internal organs. A simple administration of naked siRNA is possible and the coupling with reagents that help the siRNAs to cross the lipid envelope of the cell is possible.

There are obstacles to overcome in the development of anti-HSV siRNA drugs. Nevertheless, HSV infection treatment is feasible with siRNAs and there is a burning need to treat the billions infected and to treat with novel drugs the hundreds of thousands to millions with antiviral resistant HSV.

3 AIMS OF THE STUDY

Herpes simplex virus (HSV) causes a medical burden. In addition, novel drugs are needed to tackle drug resistance of the virus. The aim of this work was to introduce a novel approach against HSV infection.

Our antiviral approach was RNA interference using swarm of enzymatically created small interfering RNAs. Even though most epithelial HSV infections could be potentially treated with the new drug, the chosen treatment target was the HSV infection of the eye – a well established model of HSV keratitis, a disease form which has a high prevalence of drug resistant cases.

The specific aims of study were:

I Design and test for the proof of principle of the biological drug;

II Explore the innate immunity effects of the drug and HSV infection;

III Test the feasibility of the antiviral against clinical isolates of HSV;

IV Study of suitability of the biological drug in a corneal *in vivo* infection model.

4 MATERIALS AND METHODS

4.1 Quantitative PCR (I-IV)

To measure innate immunity responses, viral gene expression and DNA load, real-time quantitative polymerase chain reaction (qPCR) was used. Messenger RNA (mRNA) was first extracted and then converted into complementary DNA (cDNA). The efficacy was measured with a house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was additionally used for normalization. The subsequent analysis of mRNA quantities was done from the sample. As for viral DNA amount analysis, the DNA from the sample was extracted. Please see 4.1.1 and 4.5.2 for the RNA and DNA extraction protocols, respectively. To measure the copy numbers of analyzed genes, a real-time instrument Rotorgene (Qiagen, Hilden, Germany) was used. A standard curve was supplied for every run.

4.1.1 RNA extraction and subsequent reverse transcription

RNA extraction was done from cell cultures. The medium was removed and the cells were lysed in TRI Reagent (Molecular Research, Cincinnati, OH) and subsequently the total RNA was isolated as instructed by the manufacturer. The RNA was treated with DNase (ThermoFisher Scientic, Waltham, MA, USA) to rid the samples from possible viral DNA contaminants. For cDNA synthesis, random hexamer primers were used (ThermoFisher Scientic) with RevertAid H Minus Reverse Transcriptase enzyme (ThermoFisher Scientific). The cycle was 60’ +42°C, 10’ 70°C, hold +4°C. A total of 2% of the original cell sample was used in each qPCR run. The GAPDH count in such a run had to be over 1000 for the extraction and cDNA synthesis to be considered successful.

4.1.2 Standards for qPCR runs

All qPCR runs were performed with a six to eight step (log) dilutions of the corresponding standard, which was either a larger PCR product, a plasmid or viral DNA. Viral DNA was used as a standard when viral DNA amount was measured. For mRNA analysis a PCR product served as a standard for all but one test (α-TIF), where a plasmid served as a standard template. For the primers used to create standards and the standards used, please see **Table 3**.

Table 3. Standards and primers used to create standards for qPCR analyses.

Gene	Primers used (5'-3', upper forward, lower reverse) to create, or template used as, standard	Gene id or equivalent
US1	AAG CCC AAA TGC AAT GCT AC CAG ACA CTT GCG GTC TTC TG	NC_001806.2 (132101..133963)
UL54	GTG CCC CCA GAA CCA ATC CGG CAA AAG TGC GAT AGA G	NC_001806.2 (113735..115283)
UL29	GGT GCG GTC AAA AAT AAG GA CCT ACC AGA AGC CCG ACA AG	NC_001806.2 (58410..62054, complement)
UL27	CAC TTG GTC ATG GTG CAG AC CAC CAC CGA CCT CAA GTA CA	NC_001806.2 (53059..55795, complement)
α-TIF / VP16 / UL48	α-TIF plasmid pRB3717 [McKnight et al., 1987]	BamHI fragment F; GU734771.1
gD for HSV-1	DNA isolated from HSV-1 strain 17+	NC_001806.2
gD for HSV-2	DNA isolated from HSV-2 strain H1224	Not available
IFN-α	TGG CTG TGA AGA AAT ACT TCC G TGT TTT CAT GTT GGA CCA GAT G	NM_024013.2 NM_006900.3
IFN-β	AGA CTG CTC ATG CGT TTT CC TCC TCC AAA TTC CTC TCC TG	NM_002176.3
IFN-λ1	GAC TTT GGT GCT AGG CTT GG AAG GTG ACA GAT GCC TCC AG	NM_172140.1
IFN-λ2/3	CAG TGC TGG TGC TGA TGG GAT ATG GTG CAG GGT GTG AA	NM_172138.1 NM_172139.2
ISG54	AAG CCA CAA TGT GCA ACC TA GAG CCT TCT CAA AGC ACA CC	NM_001547.4
TLR3	ATG AAA TGT CTG GAT TTG GAC TA GTT AGC TGG CTA TAC CTT GTG A	NM_003265.2
β-actin	CCC TGG AGA AGA GCT ACG A TAA AGC CAT GCC AAT CTC ATT	NM_001101.3
GAPDH	AAT CCC ATC ACC ATC TTC CA TGA GTC CTT CCA CGA TAC CA	NM_002046

4.1.3 qPCR primers

The primers used for qPCR are presented in **Table 4**.

Table 4. Primers used for qPCR detection.

Target gene	Organism	5'-3' sequences upper sequence forward and lower reverse primer	Reference
US1	HSV-1	CAT GCG CCA GTG TAT CAA TC CGG CAG TAT CCC ATC AGG TA	III
UL54	HSV-1	GTC CTG CGC TCC ATC TCC GTC GTG CAT GAC CTG TGC	II
UL29	HSV-1	AAG CTG GTT GCG TTG GAG TTT CTG CTG AAG CAG TTC CA	I
UL27	HSV-1	TAG CTG GTG TGT TCG GTG TG GGA CGA CGG TAA ACT GCA TC	This study
α-TIF / VP16 / UL48	HSV-1	TTT GAC CCG CGA GAT CCT AT GCT CCG TTG ACG AAC ATG AA	[Broberg et al., 2003]
gD	HSV-1	CGG TAG CCC GGC CGT GTG CAT ACC GGA ACG CAC CAC ACA A	[Hukkanen et al., 2000; Mäki et al., 2015]
gD	HSV-2	ACC CAC CGC ACC ACC ATA CTC GCG ACT AGT GGT TCG CAA TGC A	[Hukkanen et al., 2000; Mäki et al., 2015]
IFN-α¤	Human	TGG CTG TGA AGA AAT ACT TCC G TGT TTT CAT GTT GGA CCA GAT G	[Peri et al., 2008]
IFN-β	Human	TCT CCA CGA CAG CTC TTT CCA ACA CTG ACA ATT GCT GCT TCT TTG	[Peri et al., 2008]
IFN-λ1	Human	GAC GCC TTG GAA GAG TCA C CTC ACC TGG AGA AGC CTC A	III
IFN-λ2/3	Human	GCC ACA TAG CCC AGT TCA AG TCC TTC AGC AGA AGC GAC TC	II
ISG54¤	Human	ACT ATC ACA TGG GCC GAC TC TTT AAC CGT GTC CAC CCT TC	I
TLR3	Human	TAG CAG TCA TCC AAC AGA ATC AT AAT CTT CTG AGT TGA TTA TGG GTA A	[Peri et al., 2008]
β-actin	Human	TTG CCG ACA GGA TGC AGA A TCA GGA GGA GCA ATG ATC ATT TGA T	[Mäkelä et al., 2006]
GAPDH¤	Human	GAG AAG GCT GGG GCT CAT TGC TGA TGA TCT TGA GGC TG	[Nygårdas et al., 2009]

¤ Annealing temperature 55 °C, others at 60 °C.

4.2 Viruses (I-IV)

The herpes simplex viruses used in the study can be divided into three categories, wild type (wt), clinical isolate and recombinant. All viruses used are presented in **Table 5**.

Table 5. Viruses used in the study.

Name of strain	HSV type	Category	Study
17+	HSV-1	Wild type	I-IV
F	HSV-1	Wild type	III
KOS	HSV-1	Wild type	III
LoxLUC	HSV-1	Recombinant, Luciferase under human CMV promoter	IV
H1211	HSV-1	Clinical isolate	III
H1215	HSV-1	Clinical isolate	III
H12114	HSV-1	Clinical isolate	III
H12115	HSV-1	Clinical isolate	III
H12117	HSV-1	Clinical isolate	III
H12118	HSV-1	Clinical isolate	III
H12119	HSV-1	Clinical isolate	III
G	HSV-2	Wild type	This study
H1224	HSV-2	Clinical isolate	This study
H1226	HSV-2	Clinical isolate	This study
H1227	HSV-2	Clinical isolate	This study
H1228	HSV-2	Clinical isolate	This study
H1229	HSV-2	Clinical isolate	This study
H12211	HSV-2	Clinical isolate	This study
H12212	HSV-2	Clinical isolate	This study

The viruses were propagated on Vero cells (African green monkey kidney cells; ATCC, Manassas, VA). For *in vitro* studies, shed viruses were used (III). Viruses in the supernatant were clarified via brief low speed (3000 relative centrifugal force [RCF]) spin and when required, subsequently concentrated further by pelleting at higher speed (20000 RCF_{max}) (I-II). For *in vivo* work, high titer viral stock was prepared from pelleted infected cells, freeze-thawed thrice, sonicated and stored in sterile 9% fat free milk in water, as described earlier [Nygårdas et al., 2013; Roizman and Spear, 1968; Syrjänen et al., 1996].

4.2.1 Clinical isolates

The clinical HSV isolates originated from anonymously archived clinical diagnostic samples obtained from herpes lesions (Department of Virology). The type of the HSV isolate was first demonstrated by an immunoperoxidase-rapid culture assay [Ziegler et al., 1988] and subsequently confirmed by a type-specific HSV DNA-PCR [Hukkanen et al., 2000]. The primers were presented in **Table 3**. These viruses were propagated on Vero cells and the stocks used in the studies were from second passage of each virus.

4.2.2 Plaque titration

Plaque titration was performed in Vero cells (I-IV). Cells in wells were infected with a dilution of the virus. After 1-2 h incubation, the viral dilution was removed and replaced with a growth medium containing human IgG. The IgG limited the spread of the virus to the cell-to-cell level. After 3(-4) days incubation in +37°C 5% CO_2 the cells were fixed with cold methanol and stained with crystal violet. Herpes plaques were visible to naked eye and counted.

4.3 Cell lines (I-III and IV)

Vero cells were used for viral propagation and titer analysis (I-IV), kept in DMEM with heat-inactivated fetal bovine serum (FBS). Cell lines derived from various human tissues were used to study *in vitro* infection inhibition. Human glioblasma-astrocytoma U373MG cells (U-251 MG; ATCC) (I-III) were maintained in high glucose DMEM supplemented with 10% FBS and 2 mM L-glutamin. Epithelial HaCaT cells (Department of Dentistry, University of Turku, Finland [Boukamp et al., 1988]) (I-III) were kept in DMEM with 7% FBS. Human telomerase reverse transcriptase-immortalized retinal pigment epithelial (hTERTRPE1, Clontech) (RPE) cells (III), were maintained in DMEM : Ham's F12 (1:1), 10% FBS, 2 mM L-glutamine, and 0.348% sodium bicarbonate. All cells were maintained in +37°C with 5% CO_2 and humidified atmosphere.

4.4 Small interfering RNA products (I-IV)

The production and concept of the siRNA swarms and how they correlate to single site siRNAs is depicted in **Figure 6**. In essence, a swarm is a pool of siRNAs

enzymatically cleaved from a hundreds of base pairs long dsRNA. Thus the amount of identical siRNAs / swarm is very low.

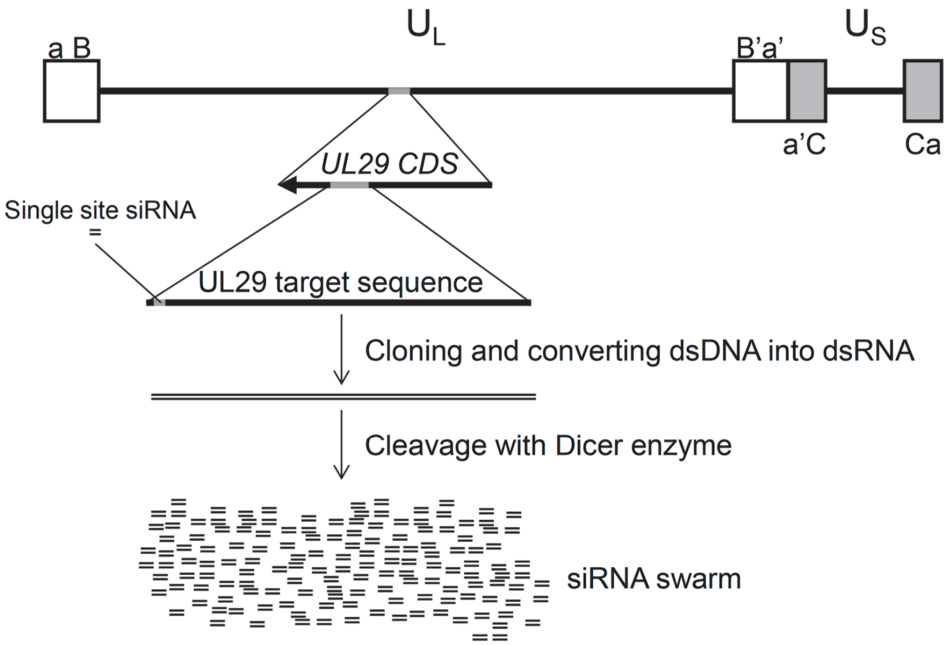

Figure 6. Schematic presentation of siRNA swarm production. The lengths of the sequence elements are in scale and each zooming step is 10x. *UL29* = HSV gene coding for ICP8. ds = double stranded. U_L = unique sequence long, U_S = unique sequence short, B/B' = repeat and its inversion flanking U_L, C/C' repeat and its inversion flanking U_S, a/a' = short few hundred base pair repeat. CDS = coding sequence.

4.4.1 RNA products used

The RNAs used in the studies are presented in **Table 6.** Throughout the studies, the enzymatically cleaved anti-HSV-UL29 and the unspecific eGFP swarms were used. To create these pools, *Giardia intestinalis* Dicer was used for cleaving. Representing the exact same sequence, a swarm consisting of shorter siRNAs was created using human Dicer, defined by an H in the swarm name. *Giardia intestinalis* Dicer was used to cleave the other swarms, UL54 and UL27 (and the swarms in ULMIX, consisting of equimolar mixture of UL27, UL29 and UL54 swarms).The synthetic anti-HSV-siRNAs were ordered from Dharmacon (Lafayette, CO, USA) and anti-GAPDH siRNA from Qiagen (Hilden, Germany).

Table 6. RNAs and RNA swarms used in the study.

(si)RNA product	Product type	Study	Target gene and its protein product	Corresponding HSV sequence[a]
UL54	Swarm	III	HSV α-gene *UL54* coding for ICP27, multi-functionary, essential. Cleaved with *Giardia intestinalis* Dicer (GD).	113947-114715
UL29	Swarm	I-IV	HSV β-gene *UL29* coding for ICP8, essential role in HSV DNA replication. Cleaved with GD.	59301-59953
UL29H	Swarm	I-II	As above, cleaved with human Dicer.	As above
UL27	Swarm	III	HSV ɣ-gene *UL27* coding for glycoprotein gB, essential for virion entry. Cleaved with GD.	54689-55207
ULMIX	Swarm	III	1:1:1 mixture of UL27, UL29 and UL54. Cleaved with GD.	All of the above
eGFP	Swarm	I-IV	No HSV or host cell targets. Cleaved with GD.	None
UL29S	Synthetic	I-II	*UL29* [Palliser et al., 2006]	59930-59948
UL29L	Synthetic	I-II	*UL29*	59925-59949
siGFP	Synthetic	I-II	No HSV or host cell targets [Caplen et al., 2001]	None
hGAPDH	Synthetic	I	Human GAPDH	None
88bp	Long dsRNA	I-II	Long dsRNA product, toxic to cells [Jiang et al., 2011]	None
GFP-tagged siRNA	Synthetic	I	Label IT RNAi Delivery Control, with no intended target and a GFP-tag attached to the siRNA	None

[a] HSV-1 strain 17+ (GenBank NC_001806.1)

The sequences had to be suitable for PCR amplification (verified with Primer3 software [Koressaar and Remm, 2007; Untergasser et al., 2012]), no known mRNA hits were allowed from human and mouse genomes (search performed with BLAST [Altschul et al., 1990]) and no matches to known miRNAs (search performed with MirBase [Griffiths-Jones, 2004]). On the other hand, the highest similarity between sequenced HSV strains was chosen. There were some similarities with other herpesviruses as well, but these were limited.

The siRNA swarms were produced by first amplifying the target sequences from HSV-1 strain 17+ purified DNA. This PCR product was then cloned into a plasmid from which the long dsRNA was produced with Replicator RNAi kit (ThermoFisher Scientific) according to the manufacturer's instructions. The dsRNAs were then sliced to siRNAs, creating a swarm, using Dicer (human or *Giardia intestinalis*) enzyme. Subsequently the swarm was purified. At first, siRNAs were purified by an anion-exchange Gen-Pak FAX column (Waters, Milford, MA, USA) (I-II) and later on (III-IV) with a more high throughput CIMac QA

column (BIA Separations, Ajdovščina, Slovenia) [Romanovskaya et al., 2013], connected to the ÄKTA purifier (GE Healthcare, Chicago, IL, USA). The sequences of the primers used to create the swarms and the single site siRNAs are shown in **Table 7.**

Table 7. Primers used for the production of the siRNA swarms and long dsRNA and the RNA molecules used.

Name	5'-3' sequences[a]	Reference
UL54-T7 sense	*TAA TAC GAC TCA CTA TAG GGC* CGT CTC GTC CAG AAG ACC	III
UL54-phi6 anti-sense	*GGA AAA AAA* CGG CAA AAG TGC GAT AGA G	
UL29-T7 sense	*TAA TAC GAC TCA CTA TAG GGA* TGA TGG CCG TAA GGG TGT	I
UL29-phi6 anti-sense	*GGA AAA AAA* CGC AAC TTT CGC AAT CAA T	
UL27-T7 sense	*TAA TAC GAC TCA CTA TAG GGC* ACT TGG TCA TGG TGC AGAC	III
UL27-phi6 anti-sense	*GGA AAA AAA* GGT GAT CGA CAA GAT CAA CG	
eGFP-T7 sense	*TAA TAC GAC TCA CTA TAG GGA* TGG TGA GCA AGG GCG AGG AG	[Aalto et al., 2007]
eGFP-phi6 anti-sense	*GGA AAA AAA* CTT GTA CAG CTC GTC CAT GCC G	
88bp-T7 sense	*CGC GTA ATA CGA CTC ACT ATA G*AT AAA CAA GTC CTT GTA	[Jiang et al., 2011]
88bp-phi6 anti-sense	*GGA AAA AAA* GAG AGA GAG CCC CCG AAG G	
UL29S/UL29.2 siRNA	5'-CUUUCGCAAUCAAUUCCAAUU-3' 3'-UUGAAAGCGUUAGUUAAGGUU-5'	[Palliser et al., 2006]
UL29L siRNA	5'-ACUUUCGCAAUCAAUUCCAACCGGUGC-3' 3'-UGAAAGCGUUAGUUAAGGUUGGCCACG-5'	I
eGFPS siRNA	5'-GCAAGCUGACCCUGAAGUUCAU-3' 3'-GCCGUUCGACUGGGACUUCAAG-5'	[Caplen et al., 2001; Nygårdas et al., 2009]

[a] The sequences corresponding to T7 and Phi6 promoter sequences are in italics.

4.4.2 In vitro siRNA delivery

After initial studies, a transfection reagent modified especially for siRNA was chosen: RNAiMAX (Invitrogen). Its suitability for transfection was tested with fluorescein-labeled siRNA (Label IT RNAi Delivery Control, Mirus Madison, WI, USA) at various cell confluency levels and various cell cultures. HaCaT cell line was used for majority of the initial tests. Being an epithelial cell line, HaCaT served both robust test setting for transfection and suitable cell type to study HSV infection and its treatment.

Experiments were performed on 60-80% confluency on 96-well plates. Toxicity of the transfection was monitored by microscopy and assessed by cell viability measurement with CellTiter-Glo luminescent assay (Promega, Madison, WI,

USA) with the measurement done with Wallac 1420 Multilabel Counter VICTOR^3 (PerkinElmer, Turku, Finland). A decrease in cell viability of at least 20% was regarded as toxicity. Doses ranging from 1 to 50 pmol of RNA were measured for changes they caused in cell viability. Cells were initially infected with various viral doses (0.001-0.1 MOI) to study the spread and replication as a function of time. A 1000 plaque forming units (PFU)/well was chosen for the experiments (0.01-0.1 MOI). Antiviral efficacy of siRNAs during various time points relative to infection, was tested. A transfection time of 4 hours prior to infection was chosen for the experiments.

4.5 Corneal infection model (IV)

For the corneal HSV infection model, young, 4-6 week-old, female Balb/c mice from the Central Animal Laboratory of the University of Turku were used under the permits ESAVI 1169 and 3029 approved by National Animal Experiment Board in Finland. The mice were kept at a 12 hours dark-light cycle with normal chow and water available *ad libitum*.

4.5.1 Anesthesia, infection and treatment

As an anesthetic, a cocktail of medetomidine (1 mg/kg) and ketamine (75 mg/kg) was given intraperitoneally (ip) with a 26G needle. After injection, the mice were placed in a new heated cage were they fell asleep. Surgical anesthesia, verified by leg pinch, followed in minutes. Subsequently, the analgesic buprenorphine dose of 0.075 mg/kg was given subcutaneously (sc) with a 27G needle to the loose skin at the back of the neck. The mice eyes were scarified with a 26G needle's open side with 15 strokes in intersecting directions (#). Following scarification, the mice were placed back into their own cage which was placed over a heating pad. Infection was performed thereafter by pipetting 10 µl of the viral (17+ or LoxLUC, 10^6 or 10^7 PFU/eye, respectively [Nygårdas et al., 2013]) dilution on each eye. One hour into the anesthesia, the mice were woken up with atipamezole hydrochloride (0.5 mg/kg) injected sc into a flank. This lower-than-textbook dose was used to enable the re-anesthesia in four hours. Despite the lower dose, the mice were up and moving about within a few minutes. The cage was removed from the heating pad at this point.

Four hours post infection, the mice were anesthetized as above and the drug was pipetted in a 7.5 µl volume on each eye. Each mouse received the same infection

and treatment on both of its eyes. After 1 h, the mice were roused as described above.

4.5.2 Follow-up and sampling

The mice were monitored daily or more frequently, when necessary due to symptoms. The health of the mice was assessed, the weight was measured and swab samples were taken from both eyes. In case of weight loss, wet food was added to the floor of the cage and the mice were checked upon more frequently. In case of encephalitis signs and/or severe weight drop (over 20%), the mice were euthanized. Please see the following paragraphs for the procedure and sampling from euthanized animals.

The swab was taken with a sterile cotton swab that was slightly moistened in cold sterile phosphate-buffered saline (PBS). Swabbing was done in a one rotating motion (of the cotton swab) over the eye in ear-to-nose direction. The swab was placed in a snap cap tube with cold 2 ml of DMEM with 5% FBS with gentamycin and amphotericin. The shaft of the swab was twisted in half so that the tube could be shut. The swabs were then processed for viral culture and HSV DNA analyses.

For mice infected with LoxLUC marker virus, luciferase signal was detected via IVIS (In vivo Imaging System apparatus, Xenogen, Caliper Life Sciences, Affligem, Belgium) [Nygårdas et al., 2013]. Mice were injected ip with 150 mg/kg D-Luciferin (PerkinElmer, Waltham, MA) with a 26G needle. The mice were anesthetized with 3.5-4% isoflurane and imaged with in the IVIS dark chamber within 5-10 minutes from luciferin injection. During imaging, a 2-2.5% isoflurane anesthesia was used. Images were acquired for 1-5 minutes.

Virus culture from the fresh swab samples. The medium-containing swab tube was vortexed and aliquoted for subsequent titration (please refer to section 4.2.2), DNA extraction (storage -20°C), and long-term storage at -70°C. The virus titration cultures were fixed 3-4 days post infection (dpi) (please see 4.2.2). Outliers were reanalyzed from long term storage samples. If continuing viral activity was suspected, a new swab sample was always obtained until the end of the experiment.

HSV DNA analysis from the swab samples was performed by first extracting DNA with a NucliSENS easyMag (bioMérieux, Marcy l'Etoile, France) machine according to the manufacturer's instructions. After DNA isolation, qPCR measurement was performed with a Rotor-Gene Q real-time instrument (Qiagen). A

1/20th of the original sample was used for the analysis. If a clear test specific DNA melting peak was observed, even a single HSV DNA copy was considered as a positive sample and used for subsequent analysis.

Sacrificing was performed with CO_2 and subsequent exsanguination via heart puncture with a 24G needle. The eyes, brain and trigeminal ganglia (TG) were removed from the dead animal. The tissue samples were placed to a tissue culture tube filled with a 5 mm stainless steel bead (Qiagen) and topped with DMEM with 5% FBS and antibiotics. The tissue was dissociated with a TissueLyser LT (Qiagen) with 50 oscillations per second for 30 seconds, spun down, and freshly titrated. To detect latent virus from the TG samples, a 5 day incubation in +37°C with 5% CO_2 was performed prior to (adding the bead for) dissociation and titration. In case of an unscheduled symptom-based euthanization, an eye swab was taken prior to terminal anesthesia and all samples, including TG, were freshly titrated.

4.6 Statistical analyses (I-IV)

All statistical tests were performed with SPSS Statistics 20 (IBM, Armonk, NY, USA) software by comparing two groups at a time using non-parametric Mann-Whitney U-test. In *in vitro* work, siRNA treatments and/or infections were compared to groups of non-transfected and H_2O-transfected cells.

4.7 Microscopy

For microscopy, Olympus Tokyo CK (Olympus Corporation Shinjuku, Tokyo, Japan) inverted microscope was used. To obtain pictures for **Figure 4,** EVOS FL Cell Imaging System was used (ThermoFisher Scientific).

5 RESULTS

5.1 Effects of RNAs on cells (I, II)

The sequences chosen for the siRNA swarm development were first checked for possible similarities in human (and mouse) genomes. Although similarities with other herpesvirus family members were found, the hits were of low similarity with only very short coverage.

The first step was to see that the cells used were capable of being transfected. A green fluorescent protein (GFP-) tagged siRNA (see **Table 6**) was used to visually detect the siRNA entry into the cells. The transfection efficiency was additionally measured by the inhibition of GAPDH expression after GAPDH-specific siRNA (study I, supplementary Figure 2). These results were standardized to β-actin expression. When it was confirmed that the transfection protocol itself was working, various doses were tested.

After initial testing, the cells were subjected to low and high doses of siRNAs to test for cell viability (**Figure 7**). As a quality control of (non-)toxicity, all siRNA batches were tested for their effect on cell viability with CellTiter-Glo assay. A long 88bp dsRNA was used as a positive control for toxic reaction. Even very high doses (50 pmols/well) of siRNAs were tolerated (relative cell viability over 0.8, with 1=non-transfected/water transfected). However, a slight drop was observed at 10 pmols/well for U373MG cells (**Figure 7A**) whereas HaCaT cells did not show this phenomenon (**Figure 7B**).

To study dose dependent reactions, 1 pmol and 10 pmol doses were studied further for their innate immunity gene expression induction at 8, 24 and 48 hours post transfection (hpt) (**Figures 8** and **9**). The 1 pmol represented a low dose whereas 10 pmols was slightly higher than standard pmol amount in siRNA screens. The 88bp long dsRNA induced robust changes in type I and III interferons (IFN-β and -λ1 [IL-29], respectively) and interferon stimulated gene 54 (ISG54) gene expression. IFN-α responses were modest all around in both U373MG (**Figure 8A**) and HaCaT (**Figure 9A**) cells. Treatment with the swarm created with human Dicer (UL29H) resulted in strongest, dose-dependent, innate immunity responses of the antiviral siRNA molecules. Longer siRNAs (25-27 nt; *Giardia intestinalis* Dicer cleaved siRNA swarm UL29, and UL29L siRNA) had similar profiles compared to each other. The short siRNA UL29S (UL29.2) had little effect on the innate responses.

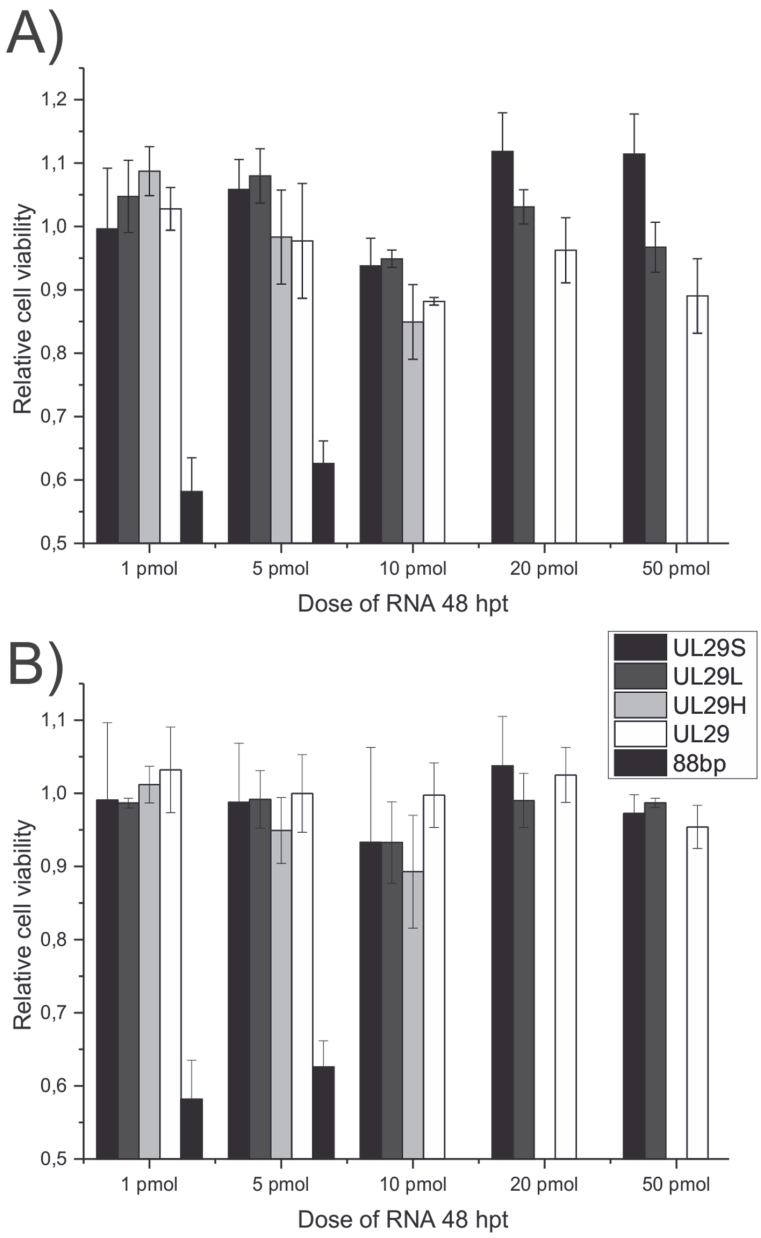

Figure 7. Cell viability changes due to siRNA treatment. A) U373MG and B) HaCaT cell viability relative to no transfection/H_2O transfection 48 hours post transfection with various RNAs and doses given to cells on 96-well plates. UL29S 21 nt siRNA, UL29L 27 nt siRNA, UL29H 21-23 nt siRNA swarm, UL29 25-27 nt siRNA swarms and 88bp an 88 nt dsRNA. 10-50 pmol dose for 88bp, and 20-50 pmol dose for UL29H not done. Mean±SEM shown. Part of the data has been published before [Romanovskaya et al 2012, study I].

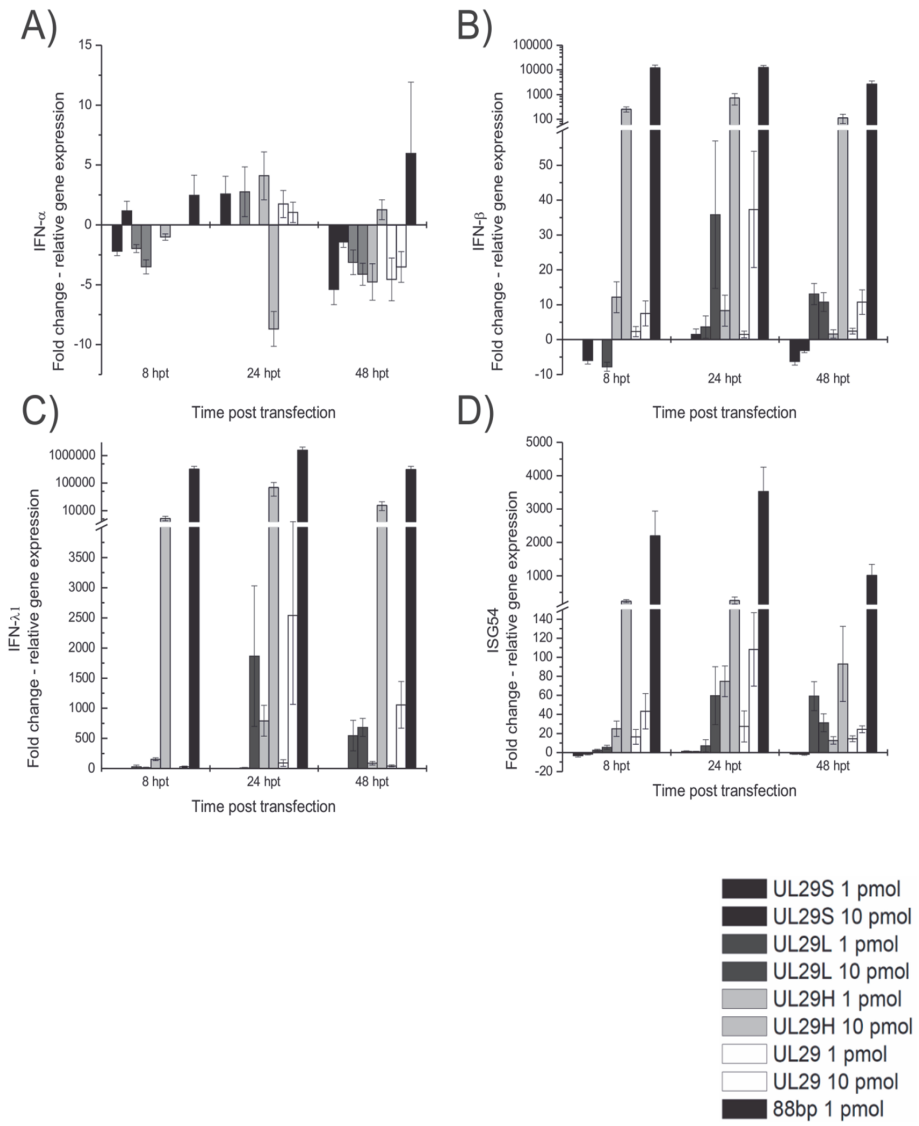

Figure 8. Innate responses to RNAs in U373MG cells. Relative expression of A) interferon α B) interferon β C) interferon λ1 (IL29) and D) interferon stimulated gene 54 (ISG54) relative expression fold change compared to no/H_2O transfected U373MG cells on 96-well plates at 8, 24 and 48 hours post transfection. The cells were transfected with the indicated dose of siRNA and samples collected at indicated timepoints. UL29S 21 nt siRNA, UL29L 27 nt siRNA, UL29H 21-23 nt siRNA swarm, UL29 25-27 nt siRNA swarm and 88bp an 88 nt dsRNA. Mean ±SEM shown.

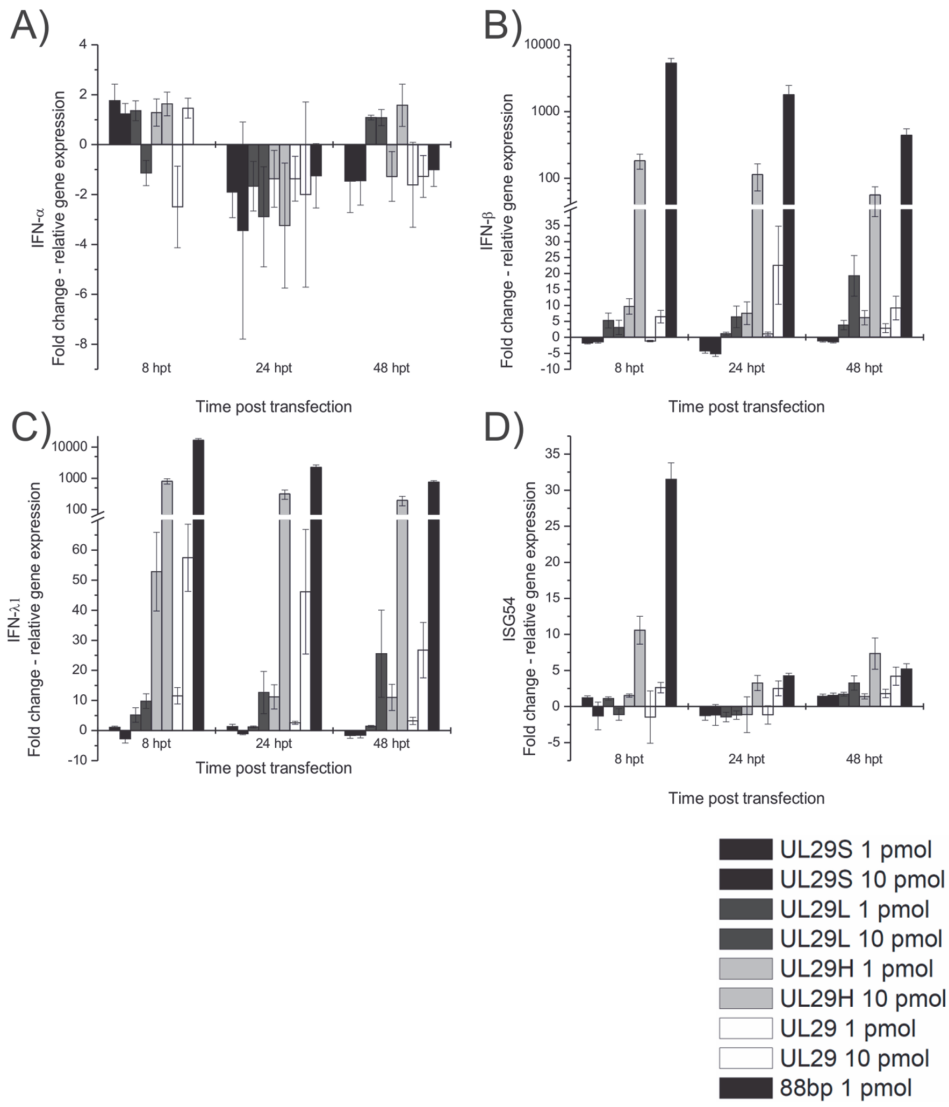

Figure 9. Innate responses to RNAs in HaCaT cells. Relative expression of A) interferon α B) interferon β C) interferon λ1 (IL29) and D) interferon stimulated gene 54 (ISG54) relative expression fold change compared to no/H_2O transfected HaCaT cells on 96-well plates at 8, 24 and 48 hours post transfection. The cells were transfected with the indicated dose of siRNA and samples collected at indicated timepoints. UL29S 21 nt siRNA, UL29L 27 nt siRNA, UL29H 21-23 nt siRNA swarm, UL29 25-27 nt siRNA swarm and 88bp an 88 nt dsRNA. Mean ±SEM shown.

In U373MG cells (**Figure 8**), over 1000-fold increase in type I and III IFN gene expression was detected for human Dicer cleaved siRNA swarm. This effect lasted for over 48 hours, whereas ISG54 gene expression started to subside by two days post transfection. However, comparing human Dicer created swarm to the toxic 88bp dsRNA, the long dsRNA did have a ten to hundred times higher gene expression induction with responses being at high levels even at late time points. The HaCaT cells (**Figure 9**) mirrored the U373MG cells for type I and III IFN responses. For ISG54 the responses were more modest and subsided faster.

Empty transfection, water transfection, and no transfection had no detectable effect on studied cells.

5.2 HSV infection inhibition *in vitro* with siRNA swarms (I-III)

5.2.1 Comparison of siRNAs of different lengths

After establishing safety features, for example the lack of possible off-target and unexpected toxic effects, of the siRNA products, their antiviral effect was studied (**Figure 10**). A non-specific siRNA swarm eGFP(G*iardia intestinalis* Dicer) was included into the testing. This swarm had no corresponding RNA matches in the host or the virus. A non-specific single site siRNA siGFP was included into the studied siRNA product list as well.

The non-specific swarm eGFP(G) had some effect in U373MG cells (**Figure 10A**) whereas in HaCaT cells (**Figure 10B**) there was no inhibition of viral shedding. The single site nonsense-siRNA siGFP had no antiviral effect regardless of the cell line used. As for the various HSV-specific siRNA products, the siRNA swarms created with both human and *Giardia intestinalis* Dicer had the strongest antiviral effect. By visual inspection (data not shown) and cell viability (**Figure 7**) the 88bp dsRNA killed the cells quicker than HSV was able to replicate.

As swarms created with human Dicer and *Giardia intestinalis* Dicer both had comparable antiviral properties (**Figure 10**), but the human Dicer swarms had a higher decrease on the cell viability and higher induction of innate responses (**Figures 7-9**), *Giardia intestinalis* Dicer was chosen as the method of choice for the production of novel target sequences against HSV.

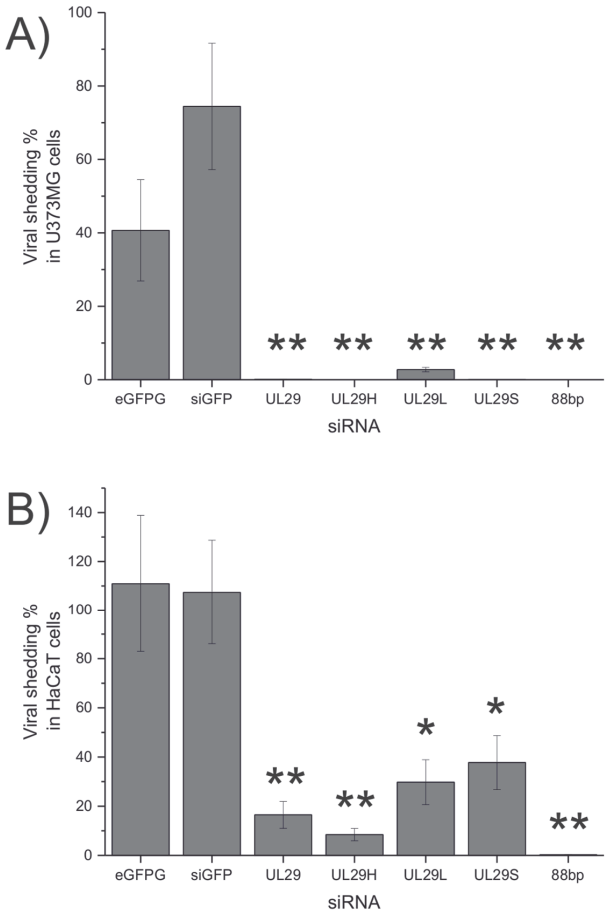

Figure 10. Viral shedding proportion from treated and HSV-1 infected A) U373MG and B) HaCaT cells. Viral shedding inhibition (%) in relation to no /H_2O treatment. Viral shedding was measured by plaque titration of the supernatant two days post 1000 PFU infection. UL29S 21 nt siRNA, UL29L 27 nt siRNA, UL29H 21-23 nt siRNA swarm, UL29 25-27 nt siRNA swarms and 88bp an 88 nt dsRNA. *=$p<0.05$ and **=$p<0.01$ compared to no/H_2O treatment. Mean±SEM shown.

5.2.2 Comparison of the anti-HSV-siRNA swarms

After showing the antiviral efficacy of UL29, new anti-HSV-siRNA swarms targeting other HSV genes expressed at different phases of infection were studied. HSV genes *UL54* and *UL27* were chosen as additional targets against HSV; the siRNA swarms being named UL54 and UL27, respectively. UL54 is an immedi-

ate early gene coding for ICP27, an essential multifunctional regulatory protein. UL27 is expressed at the late stages of infection and encodes for glycoprotein B (gB), which is an essential component in virion entry. In addition, a mixture of these HSV-specific swarms, including the UL29, was also used (ULMIX) (**Table 6**). Type I and III interferon and TLR3 gene expression in U373MG, HaCaT and RPE cells were studied for their responses to siRNA swarms and HSV infection (see **Table 12** in Discussion section 6.1.1).

The above tested swarms were subjected for proof-of-principle antiviral testing. Each swarm was tested for its capability to inhibit its own target gene(s) (**Table 8**). The proportion of inhibition of viral mRNA production was over 99.9% for the swarm targets. All anti-HSV siRNAs were effective ($p<0.001$) against HSV-1 strain 17+ and were significantly ($p<0.05$) more effective than eGFP in U373MG cells.

Table 8. Inhibition of target gene(s) in U373MG cells. U373MG cells were treated with 10 pmol/well of the indicated swarm and subsequently infected with HSV-1 strain 17+. Samples were collected 44 hpi/48 hpt and mRNA expression was analysed.

Swarm / measured target gene	Target gene inhibition (%) HSV-specific vs eGFP-pool
UL54	99.54
UL29	99.98
UL27	99.90
ULMIX	99.65 – 99.83 – 99.89[a]

[a] Inhibition of UL54, UL29 and UL27, respectively.

For a drug to be suitable in antiviral development, it is of importance to test the candidates against viral strains from clinical isolates. This is especially important for RNAi based drugs, since there is genetic variation between viral strains. When challenged against multiple HSV-1 strains, including wild type and clinical isolates, the most effective swarm was UL29 with an average inhibition rate of over 92% (**Table 9**). The other swarms were effective as well but with ULMIX being to only other drug to reach overall inhibition of over 90%. There was some antiviral effect in the unspecific swarm, but this was surpassed by all anti-HSV-swarms.

Table 9. The antiviral efficacy (inhibition of viral shedding) of the siRNA swarms. The effect of the swarms was measured in comparison to no/H_2O transfection controls. All shown strains are of HSV-1.

U373MG cells	HSV-1 17+	HSV-1 F	HSV-1 KOS	H1211	H1215	H12114	H12115	H12117	H12118	H12119	Average inhibition (%)
eGFP	68.7±8.7	**91.5**±3.3*	**97.3**±0.8**	77.2±6.8*	56.6±13.0	18.6±31	***81.0***±3.7	53.7±13	69.0±6.0*	45.0±28	65.9±13
UL54	**90.9**±3.4**	**99.3**±0.2**	**98.0**±1.2*	**97.1**±1.2**	**97.6**±1.1**	**98.0**±0.7**	**94.3**±1.9**	**97.9**±1.2**	**94.4**±2.8**	**83.6**±9.3*	**95.1±2.3**
UL29	**99.7**±.2**	**99.9**±0.1**	**99.99**±0.0 **	**99.8**±0.1**	**99.4**±0.5**	**99.5**±0.2**	**99.8**±0.1**	**98.8**±0.8**	**98.3**±1.0**	**98.1**±1.0**	**99.3±0.4**
UL27	**97.1**±0.8**	**99.4**±0.2**	**99.4**±0.2**	**84.6**±5.2*	**93.9**±1.2**	**98.7**±0.2**	**97.2**±0.8**	**96.9**±1.1**	**96.9**±1.0**	*75.6*±4.7	**94.0±1.5**
ULMIX	**95.7**±1.5**	**99.7**±0.1**	**99.7**±0.1**	**96.3**±1.0**	**92.2**±3.6*	**98.4**±0.7**	**93.4**±3.3**	**93.1**±3.2**	**97.9**±0.7**	93.1±2.1**	**96.0±1.6**

RPE cells	HSV-1 17+	HSV-1 F	HSV-1 KOS	H1211	H1215	H12114	H12115	H12117	H12118	H12119	Average inhibition (%)
eGFP	72.5±9.6	*67.2*±12	58.3±16	-262.8±88	-25.1±34	-13.0±36	16.0±28	69.2±7.7	13.9±22	-35.5±37	-4.0±29
UL54	**74.1**±7.1**	**94.5**±2.4**	**99.1**±0.3**	*46.2*±12	76.8±9.4*	**84.9**±2.1**	***85.5***±2.1	77.0±11	60.2±14	-114.7±69	58.4±12
UL29	**99.3**±0.2**	**99.8**±0.1**	**96.2**±1.2**	**86.5**±4.7**	**95.5**±1.3**	76.2±8.3*	**96.5**±0.9**	**99.4**±0.1**	**86.6**±6.0**	*61.5*±11	**89.8**±3.4
UL27	**83.0**±4.1**	**99.3**±0.2**	***81.6***±8.6	**83.8**±5.4**	**85.1**±5.4**	**97.8**±0.5**	**89.0**±1.2*	**81.6**±8.8	**90.2**±4.2**	26.4±21	**81.8**±5.9
ULMIX	**86.2**±3.4**	**99.6**±0.1**	**99.5**±0.1**	**92.0**±1.4**	**98.1**±0.3**	**95.9**±0.5**	**95.6**±1.6**	**94.1**±1.8*	**95.3**±1.7**	*62.7*±10	**91.9**±2.1

HaCaT cells	HSV-1 17+	HSV-1 F	HSV-1 KOS	H1211	H1215	H12114	H12115	H12117	H12118	H12119	Average inhibition (%)
eGFP	64.7±12*	74.7±4.7	-8.5±55	20.5±18	**95.8**±0.8*	*56.6*±17	55.0±19	*72.8*±3.2	***84.1***±7.1	**80.5**±4.0	61.3±14
UL54	**97.5**±0.9**	*78.5*±9.0	58.3±20	-2.2±51	***92.0***±3.6	*55.3*±23	73.8±14 *	*77.5*±9.4	78.8±16*	**81.5**±6.4	69.1±23
UL29	**99.1**±0.3**	**94.7**±3.1**	**94.3**±3.3*	**87.0**±3.5**	**95.7**±2.4**	*60.3*±15	**82.0**±10**	**93.6**±1.4*	**87.0**±6.5*	**91.2**±2.0*	**88.5**±4.8
UL27	**97.4**±0.6**	*77.1*±9.4	**84.9**±9.5*	*66.3*±20	**96.6**±1.1*	*57.3*±18	3.1±75	**93.2**±1.7*	34.5±33	66.8±5.7	67.7±27
ULMIX	**97.1**±1.2**	**96.3**±1.3**	***87.6***±6.7	*59.0*±20	**97.3**±0.9**	74.4±10 *	**86.8**±4.4**	**88.3**±6.3*	**84.2**±7.6*	78.6±8.6	**85.0**±6.7

* p≤0.01 ** p≤0.001 Lower efficacy indicated by italics and underlining (p<0.05) Over 80% inhibition bolded ±SEM is shown

All HSV specific swarms remained effective against clinical HSV-1 isolates. There were, however, some viral strains capable of replicating to some extent even in the presence of certain HSV specific swarms. This depended on the cell line in question. UL29 and ULMIX were the most effective swarms. UL29, with the most overlap with HSV-2 sequences, was studied against HSV-2 strains, both wild type (HSV-2 G) and clinical isolates (**Figure 11**). All clinical isolates were not readily replicating in the given 2-day time frame (**Figure 11A**), resulting in a couple of non-significant findings in UL29 HSV-2 inhibition capabilities as there was little to inhibit. However, the HSV-1 specific UL29 remained highly active against HSV-2 as well.

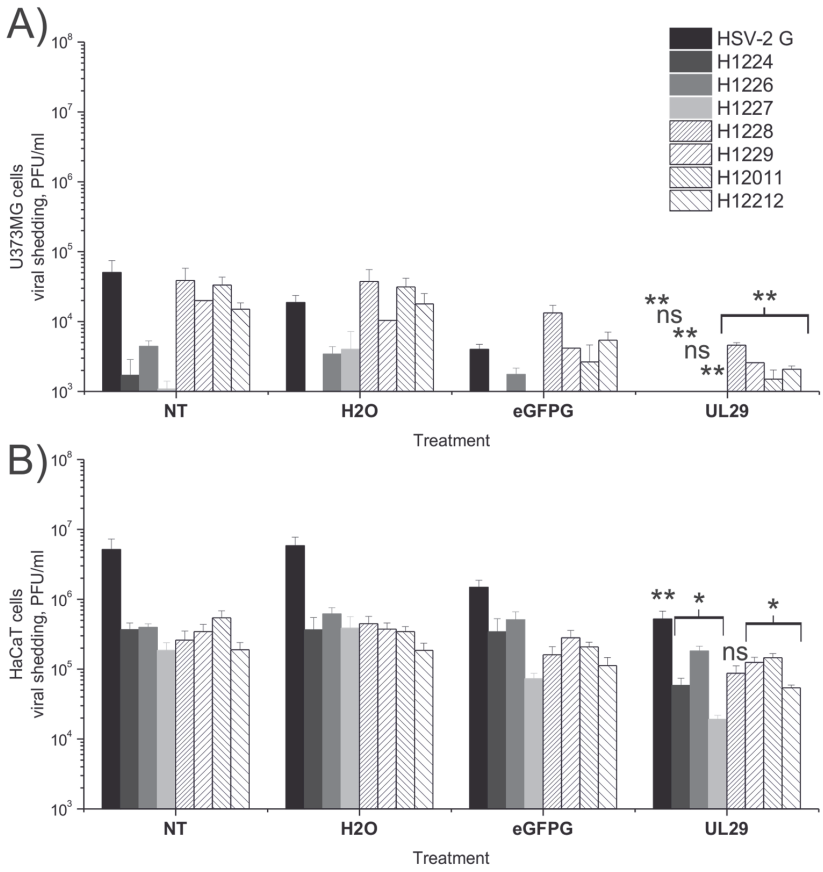

Figure 11. Antiviral properties of UL29 swarm against HSV-2. UL29 siRNA pool against 1000 PFU infection of laboratory wt strain G and clinical isolates (H1224, H1226, H1227, H1228, H1229, H12211 and H12212) of HSV-2 in A) U373MG and B) RPE cells. Supernatant samples titered on Vero cells 48 h post treatment, 44 h post infection * = $p<0.05$ and ** = $p<0.01$ against no treatment (NT)/H_2O treatment, ns = non-significant. UL29 and eGFPG 25-27 nt siRNA swarms. Average ±SEM shown. The y-axes have the same range in A) and B).

5.3 Corneal infection treatment with enzymatically created siRNA swarms (IV)

In the *in vitro* studies (I-III) we found potential antiviral swarms against HSV. These swarms targeted HSV genes *UL27, UL29* and *UL54*. Anti-UL29 swarm had the highest antiviral effect combined with the low innate immunity gene expression induction. These qualities made it the top candidate for *in vivo* testing.

5.3.1 Encephalitis model

Corneal HSV infections were done in young female Balb/c mice. After infection, the mice were treated with swarms or vehicle (PBS). A dose of 10^6 PFU / eye of HSV-1 strain 17+ resulted in severe infection. Only 20% of the mice were not subjected to euthanization due to weight drop and encephalitis signs by day 8 post infection (and please see later **Figure 13**). The unspecific swarm eGFP did not increase survival. When treated with UL29, the survival clearly increased being 60% (p=0.063 compared to vehicle treated mice, please see study IV Figure 1). The overall condition (measured by weight % compared to start of the experiment) of the animals was additionally improved when treated with UL29 compared to vehicle and unspecific siRNA swarm treatments (**Table 10** and IV Figure 1B).

Table 10. Changes in weight after HSV-1 corneal infection. Balb/c mice were infected with 10^6 PFU / eye and 4 hours post infection treated once with vehicle (PBS), unspecific eGFP or HSV specific UL29 swarm (250 pmol/eye).

	Days 1-2			Days 3-5			Days 6-7 post infection		
Treatment	**PBS**	**eGFP**	**UL29**	**PBS**	**eGFP**	**UL29**	**PBS**	**eGFP**	**UL29**
PBS	-	ns	ns	-	ns	*	-	ns	*
eGFP		-	ns	ns	-	*	ns	-	*
UL29	ns	ns	-	*	*	-	*	*	-

ns = non-significant * = $p<0.05$

No changes were seen in viable viral shedding from the eye at early, intermediate and late stages of the disease. However, the UL29 treated mice had significantly lower HSV DNA load in the eye swabs at 6 dpi of infection compared to PBS treated mice (study IV Figure 2B). Upon euthanasia, the eyes and trigeminal ganglia (TG) were freshly dissociated and titered on Vero cells to determine the replicating viral load in the tissue. The unspecific swarm was able to inhibit TG viral load, but there was no difference in the peripheral sample, as the viral load

in the eye was comparable to PBS treated mice. However, the UL29 strongly inhibited viral load both in the eye and TG ($p<0.01$ and $p<0.05$ respectively, study IV Figure 2C-D). In the eyes, UL29 significantly inhibited replication competent viral load compared to the unspecific swarm as well ($p<0.01$). All brains collected from euthanized mice had detectable viral replication (**Table 11**). As for explant cultures, UL29 was able to inhibit viral spread to the TG in the wt infected mice. However, in the LoxLUC infected mice, there was no reduction in the viral TG penetrance with the UL29 swarm. The unspecific swarm had a reductive effect on the positive TGs. There was, however, no differences seen in the viral titer loads between treatments (study IV, Figure 3).

Table 11. Replication competent virus from tissue samples.

	Replication competent virus (positive / tested samples)					
Virus	**HSV-1 wt (17+)**			**LoxLUC**		
Treatment	**PBS**	**eGFP**	**UL29**	**PBS**	**eGFP**	**UL29**
Brains of mice with encephalitis symptoms	4/4	4/4	2/2	n/a	n/a	n/a
TG explant culture	2/2	2/2	4/6	5/10	3/10	6/10

n/a = not applicable; all mice in the group survived and did not display signs of encephalitis.

5.3.2 Peripheral infection model

To study the infection with live imaging, we used HSV-1 17+ based LoxLUC virus [Nygårdas et al., 2013] constructed with BAC-technique and having a luciferase phCMV cassette (**Table 5**, please see 4.2). This virus was slightly attenuated and did not invade CNS as readily as HSV-1 strain 17+. Active viral presence was detected from the periphery, but not from the CNS with IVIS imaging (**Figure 12**). Viral activity was detected usually for 5 dpi. In rare cases (see panels on the left in **Figure 12**), peripheral spreading was detected. There was, however, no detectable viral shedding from the new areas. New lesions as such were not visible, but swelling was present.

None of the mice succumbed to the LoxLUC infection (**Figure 13A**). The mice did not experience weight drop either (**Figure 13B**) as they were comparable to non-infected mock mice. In addition to luciferin-luciferase signal (**Figure 12**), there was viral replication detected from the eye swabs. When treated with UL29 the viral shedding (PFU) and viral DNA load were downregulated at the peak of the disease (3-5 dpi; $p<0.05$, study IV, Figure 3). The unspecific eGFP pool had some effect as at 4 dpi there was a significant drop in viral shedding (please see IV Figure 3 for details). There was not, however, difference detected in the daily viral DNA load when treated with eGFP swarm. All in all the UL29 was broadly effective in inhibiting viral shedding and symptoms caused by HSV.

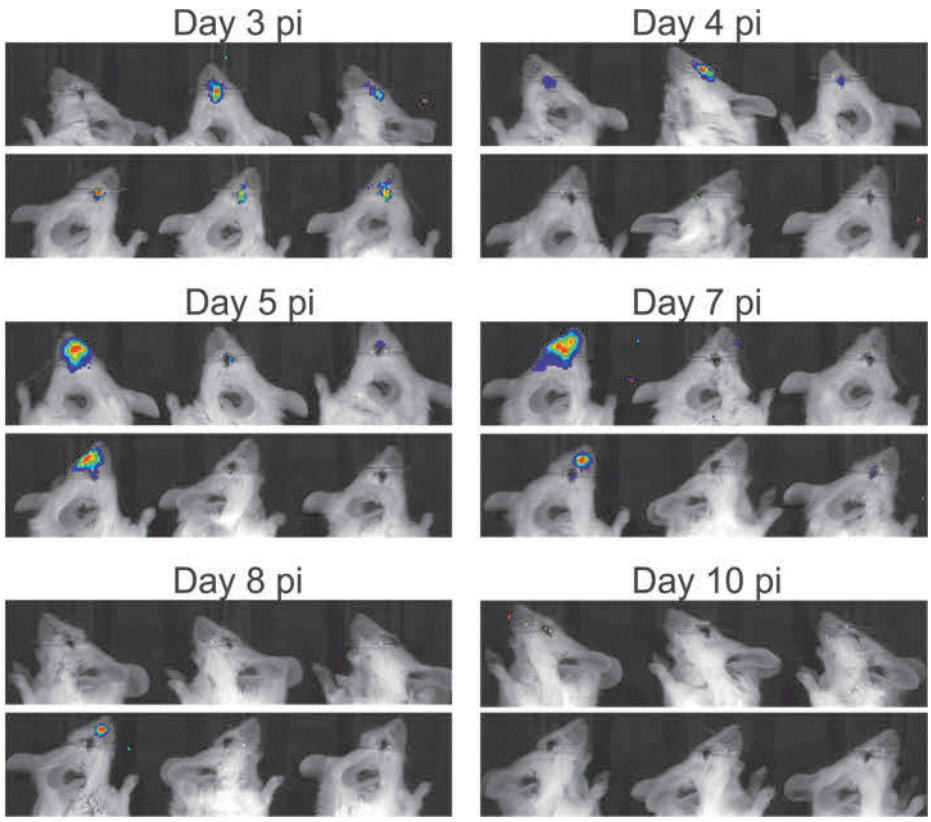

Figure 12. LoxLUC corneal infection followup with IVIS imaging. The HSV infection of mice was illustrated by the activity of the viral transgene luciferase (LUC). The procedure involved ip injection of luciferin and measurement of light signal from LUC oxidization of luciferin with ATP. Measurement and photography were done by IVIS imaging under isoflurane anesthesia (3-10 dpi). The viral activity can be seen as a light signal (intensity increasing from purple to red).

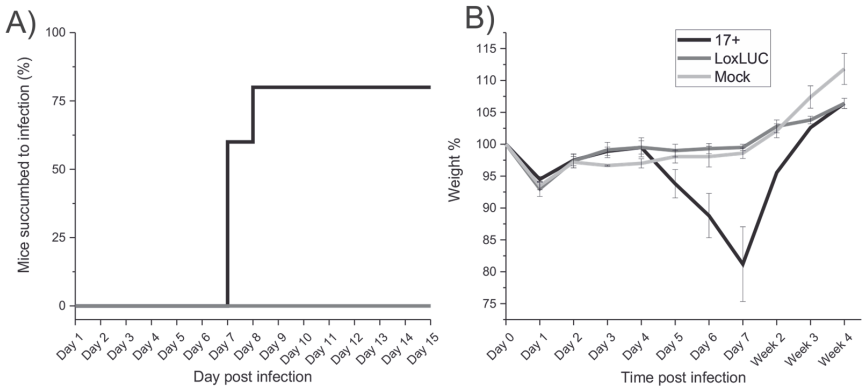

Figure 13. Survival after corneal HSV challenge. A) Mice were subjected to HSV strain 17+ (10^6 PFU/eye) or LoxLUC (10^7 PFU/eye) corneal infection and their survival was followed. B) The weight %, relative to the weight of day 0, of the mice was recorded daily and followed for the duration of the experiment. The non-infected mock mice received the same treatments (ie. anesthesia, scarification) as the infected, but without the virus.

6 DISCUSSION

6.1 Cells treated with siRNA swarms (I-II)

The proof of principle of applicability of the siRNA swarm in HSV infection control was investigated in study I. The siRNA production kinetics and purity were studied (study I Figure 2 and data not shown). The purity was considered crucial, as residual amounts of longer dsRNA molecules could result in a steep cell viability drop. As reported by [Jiang et al., 2011], longer dsRNAs, and especially the 88 bp dsRNA, cause a strong innate immunity reaction. We saw in our experimental models, that already 1 pmol of the 88bp caused a drop in cell viability, which was considered toxic. The effect was also seen in microscope inspection. Thus after production of each drug batch, the possible toxicity was measured via cell viability. Somewhat dose-dependent reaction was observed (**Figure 7**). However, even high doses of the specific siRNA swarm, up to 50 pmols/well, were well tolerated. There was no significant difference between the various siRNA products, investigated in our cell viability assays.

To study the effects of the siRNAs more closely, mRNA expression of innate immunity markers were studied. Here, a clear dose-dependent response was seen (**Figures 8** and **9**). In concordance with previous findings by others [Reynolds et al., 2006], the longer single site (anti-HSV-)siRNA caused higher innate immunity responses. However, on the contrary, the length of the siRNA was not a factor with the siRNA swarms. The shorter siRNA containing swarm created with human Dicer (UL29H; 21-23 nt) induced much higher production of type I and III and ISG genes compared to *Giardia intestinalis* Dicer cleaved UL29 (25-27 nt). Approximately equal responses were seen to 1 pmol of human Dicer cleaved swarm and 10 pmols of *Giardia intestinalis* swarm. The 10 pmol dose of swarm cleaved with human Dicer led to high increase in the type I and III interferons and interferon stimulated gene expression responses (**Figures 8** and **9**). Different cell lines can have different reaction to RNA products [Reynolds et al., 2006], and similarly the siRNA products used here had variable (off-target) gene expression induction profiles depending on the cell lines. Dicer has been suggested to be a chaperone-like protein, guiding single strand RNA for annealing with complementary RNA [Kurzynska-Kokorniak et al., 2016]. It is feasible that the Dicer loading of longer siRNAs could play a major role in the low interferon induction. Moreover, as the amount of siRNAs containing the same sequence within a swarm is low, the amount of possible toxic siRNAs due to sequence [Fedorov et al., 2006], is low as well. Moreover, the Dicer-substrate UL29 swarm and swarm of canonical siRNA length, UL29H, covered the same sequence but

had different innate immunity gene expression induction profiles. This interesting discrepancy between these swarms cannot therefore be sequence specific but is caused by another factor(s), of which Dicer loading is very likely candidate. It would be of interest to pursue this phenomenon further. However, from a drug development point of view, these results already tipped the scales in favor of the Dicer-substrate UL29 swarm rather than the UL29H. Of these two candidates both had same antiviral effect whereas the non-Dicer-substrate UL29H swarm induced interferon and interferon stimulated gene expressions much more clearly.

The findings with the synthetically produced single site siRNAs UL29S and UL29L (21 and 27 nt long respectively) were in canon with previous findings by others [Bhuyan et al., 2004; Palliser et al., 2006] as their induction of interferons and interferon stimulated genes was relatively low to non-existent. The toxic 88bp dsRNA -induced innate immunity gene expression was on a different level when compared to other RNA products. They were, however, in line with previous and following findings [Jiang et al., 2011; Jiang et al., 2015]. The amount of induced gene expression of *IFN-α*, *IFN-β*, *IFN-λ1* and *ISG54*, was cell type-dependent, but nevertheless both U373MG and HaCaT cells reacted in the same manner (**Figures 8** and **9**, respectively). The responses (the measured gene expression) were detected already at 8 hpt. However, they were detectable even at 48 hpt. This paved way for studies of the interplay of the virus and the biological drugs. Interferons as such are used against viral infections, especially when there are no other drugs available, for example against certain hepatitis viruses [Lappalainen and Färkkilä, 2016]. Interferon induction caused by the RNA products could facilitate antiviral responses. And, indeed, in some cases there was an antiviral effect observed at the same time as interferon gene expression increase. However, without question, the highest specific anti-herpes effect was seen from sequence based RNA-interference reactions from the HSV-specific siRNAs.

To study the antiviral effect, an *in vitro* setting that resembled an actual *in vivo* infection was used. In a natural infection, only a small portion of cells are infected with the virus spreading from one cell to another. At the same time the cells are communicating with each other. Depending on the virus, the amount of information and priming to infection can most likely vary. Priming with immune system molecules, such as interleukin 27, leads to inhibition of infection [Heikkilä et al., 2016a]. Moreover, HSV infection leads to exosome production, which in turn has an effect on the infection [Han et al., 2016; Heikkilä et al., 2016b; Kalamvoki et al., 2014]. In our *in vitro* experimental setting, the drug would be in both cells that have and in those that do not have the virus. Therefore a relatively low initial viral dose and a time point of 2 dpi was chosen. This was to mimic the microenvironment of an actual infection. In addition, the drug dose

(pmol) was set to mimic same situation and the dose was set for per well, not per cell. The drawback of this experimental setting was that all of the cells were not in uniform situation and viral replication was at various stages at a given time.

All in all, the siRNA products were found to efficiently inhibit viral shedding ($p<0.05$) (**Figure 10**). The strongest inhibitor of HSV was the 88bp dsRNA. However, it cannot be taken as a suitable candidate for drug development as it is a desirable feature for the drug to leave the host alive, but the 88bp dsRNA did kill virus-infected cells non-selectively. This finding points out another reason why it is important that an RNA-product does not cause too high innate immunity induction, as for example with UL29 compared to UL29H. For some target viruses a need might arise to use an RNA swarm-drug simultaneously with administered interferon. Here, a too strong of an innate immunity inducer swarm might alongside with the interferon cause undesired side effects.

6.1.1 Interplay of treatment and infection (II-III)

HSV induced innate responses, namely type I and III IFN and IFN -stimulated gene (here *TLR3*) expressions, in the host cells. This response was cell type-specific (**Table 12**). For example U373MG cells had very low response to HSV infection in this setting whereas HaCaT and RPE cells had strong responses, both up and downregulation of genes. However, the treatment of infection with siRNA swarms had an effect on the responses in U373MG cells. This was important to know, as some cell lines have very limited ability to react with and transcribe IFN-genes [Desmyter et al., 1968]. However, these U373MG cells had the capability to react with and transcribe these innate response genes. The siRNAs had an effect on the other cell lines as well. Type I and III interferons were upregulated. TLR3 gene expression was upregulated in unison to swarm treatments, being an interferon-induced component of the innate immune system. These differences in innate immunity gene expression profiles are likely causative factors on the variable effects of the siRNA swarms, especially the unspecific eGFP swarm. Here the HSV effect was studied for strain 17+, but different clinical isolates have different effects on cells (Lehtinen et al. unpublished results).

Table 12. Innate immunity gene expression responses to HSV-1 infection and siRNA swarms. The results represent at 44/48 hours post infection / post transfection.

			Responses										
Cell line	Gene	Base-line	HSV	to HSV and/or siRNA swarm									
U373MG	Type I IFN	-/+	0	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑
	Type III IFN	-/+	↑	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑	↑↑	↑↑
	TLR3	+	0	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
HaCaT	Type I IFN	+/-	↑	↑↑	↑↑↑	↑↑	↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑	↑↑↑
	Type III IFN	+	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑
	TLR3	++	↓↓	↑↑	↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
RPE	Type I IFN	+	↑↑	↑	↑↑↑	↑	↑	↑	↑↑↑	↑	↑↑↑	↑	↑↑↑
	Type III IFN	0	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑
	TLR3	++	↓↓↓	↑	↓	↑	↑	↑	0	↑	0	↑	↑
	Variables												
	HSV	-	+	-	+	-	+	-	+	-	+	-	+
	eGFP	-	-	+	+	-	-	-	-	-	-	-	-
	UL29	-	-	-	-	+	+	-	-	-	-	-	-
	UL54	-	-	-	-	-	-	+	+	-	-	-	-
	UL27	-	-	-	-	-	-	-	-	+	+	-	-
	ULMIX	-	-	-	-	-	-	-	-	-	-	+	+

+ positive - negative 0 = no effect / no baseline expression
↑/↓ = tendency of increase / decrease of gene expression
↑↑/↓↓ = tenfold increase / decrease of gene expression
↑↑↑/↓↓↓ = over 100 fold difference
HSV = herpes simplex virus infection, eGFP, UL29, UL54, UL27 siRNA swarms targeting corresponding gene, ULMIX is a 1:1:1 mixture of UL29, UL54 and UL27 swarms.

Response pathways to HSV infection can be cell type-specific [Malmgaard et al., 2004]. Type I and type III interferons can be induced by different routes [Iversen and Paludan, 2010]. HaCaT and RPE cells had similar profiles with type I and III interferon gene expression increase and TLR3 gene expression decrease due to HSV infection (**Table 12**). As mentioned above, all cell lines reacted to the given siRNA swarms. For IFN responses, there was an additive effect when the cells were both infected and treated. For TLR3 response, the swarms inhibited HSV-induced downregulation of the gene. The only clear exception to these phenomena was UL29. Viral inhibition was surely a contributor to this factor. In addition,

it is likely that the targeted viral gene and its expression phase in a lytic infection played a role in the innate immunity gene expression responses. Inhibition of *UL29* encoding protein, the ICP8, the protein responsible in part for DNA replication, should lead to halt/delay in viral DNA accumulation. This in turn could result in lighter cellular response. Why would this lead into different profiles compared to the other HSV targeting swarms? UL54 siRNA swarm had an additive effect on the innate responses, but then the swarm did not inhibit viral replication as strongly as UL29. On the other hand, UL27 siRNA swarm target, *UL27,* is expressed later than *UL29* thus not inhibiting viral DNA replication as such.

In UL29 treated cells, the infection influenced only little the interferon gene response. The induction of interferon and TLR3 gene expression due to siRNA swarms, including non-specific eGFP, could influence infection inhibition beneficially in a whole organism. TLR3 plays an important role in HSV infection [Peri et al., 2008; Reinert et al., 2012; Zhang et al., 2007]. An immunostimulant like mechanism of action of an unspecific swarm was seen, with some inhibitory effect on viral shedding in some settings against certain virus strains. Immunostimulus of an siRNA product has been found to have a favorable effect on antiviral activity, at least against Semliki Forest virus [Gantier et al., 2010]. However, the immunostimulus of a drug could also result in an increase of adverse effects.

In general, the siRNA effect and the HSV effect were additive on the innate immunity gene expression. If HSV and RNA product both induced IFN gene expression, the expression was higher for the combination than either individually. In some cases, if HSV response caused a downregulation and the swarm caused an upregulation of certain gene expression, the detected net effect remained near zero. When the virus and the swarm caused a positive reaction, the reaction was additive. The only exception to this was UL29, where infection and the swarm did not cause an additive effect. Swarm targeting *UL29* had the highest likelihood of not causing adverse effects and at the same time had the strongest antiviral effect (**Table 9**), being the most favorable combination. These features of the UL29 swarm made it a top candidate for further drug development.

6.2 Efficacy of the antiviral swarm *in vitro* (I-III)

The HSV-specific siRNA swarms were produced using HSV-1 17+ DNA as a template. Thus, the swarms were effective against HSV-1 strain 17+ (**Table 8**). As the swarms had an effect on the innate responses and the treatment was given prior to infection, it was interesting to see that the unspecific siRNA swarm had

some effect against HSV. The antiviral effect of interferon-inducing siRNA is even more prominent, the earlier the treatment is given even, especially in HaCaT cells [Backman, 2014]. To place the swarms to actual test, various HSV strains, both clinical field isolates and wild type HSVs were used (**Table 9**). When studying the antiviral effect against clinical isolates of HSV, two swarms emerged: the UL29 and ULMIX were most broadly effective against HSV in the various cell lines. UL29 had the most resemblance of the swarms to HSV-2 genome, and was found effective against HSV-2 strains, both wt and clinical isolates (**Figure 11**). Moreover, there was no evidence of quick resistance building against UL29 (study III, Figure 4). Furthermore, it is unlikely that a virus can form resistance even in the long run to all of the siRNAs in the swarm, as the targeted area is hundreds of bps long.

The UL29 swarm had a more beneficial innate immunity gene expression induction profile than ULMIX. At same pmol-dose, the UL29 caused much milder innate immunity induction profiles than ULMIX. Compared to HSV infection, the ULMIX treatment caused an significant upregulation of interferon gene expression (**Table 12** and from study III Figures 1-2), raising concerns about possible side effects in whole organisms.

6.3 *In vivo* efficacy of the swarms against HSV (IV)

Based on the *in vitro* findings, anti-UL29 swarm was chosen for the *in vivo* treatment approach. As HSV eye infection causes serious medical burden, with the additional problem of frequent antiviral chemotherapy-resistant HSV infection cases, HSV keratitis was used as a model for infection and treatment. The fact that the first antiviral RNA interference like drug to reach the market was against CMV eye infection (Vitravene, DNA oligo) [Crooke, 1998], is also an encouragement. In contrast to our *in vitro* experiments, in the *in vivo model*, the treatment was given post infection and without transfection reagent. The siRNAs swarms were delivered as eye drops in PBS.

A single 250 pmol/eye dose of UL29 swarm was able to increase mouse survival by three fold. This topical siRNA swarm treatment was effective, while, in contrast, in an antibody-mediated antiviral approach against corneal HSV, no effect of topical treatment was found [Krawczyk et al., 2015]. The increase of survival was similar to that of an HSV-2 intravaginal challenge treated with a synthetic siRNA product [Palliser et al., 2006], and could perhaps be increased further with the combination of ACV to the treatment [da Silva et al., 2016]. In addition to survival, symptoms were alleviated with the swarm (**Table 10)** and viral load in TG was limited (**Table 11**). The unspecific swarm did also somewhat inhibit vi-

ral load in TG. Immune system activation has shown to be beneficial against HSV in previous studies [Taylor et al., 1989] and in our *in vitro* work. However, eGFP swarm did not increase survival nor did it alleviate symptoms (**Table 10**).

The Balb/c mice were susceptible to corneal HSV challenge with the wt virus whereas the attenuated marker virus resulted in an attenuated, peripherally limited, infection (**Figures 12** and **13**). Even though rare, an interesting peripheral viral spread without site-specific viral shedding was observed (**Figure 12**) for the marker virus LoxLUC. The spread was most likely via neurons as has been demonstrated previously by others [Balliet et al., 2007; Halford et al., 2004].

The further the infection proceeded from the periphery, the more the weight of the mice started to plummet by 5 dpi. Brains of these mice had detectable replicating virus. For the surviving mice the weight drop was not so drastic but observable. For the UL29 treated mice, the weight loss was significantly reduced (**Table 10**), and the tissue viral load was also reduced in the UL29 treated, euthanized mice (**Table 11**). Not all mice survived the viral challenge even though they were treated. A more frequent dosing might be able to protect the mice better. However, when the virus reaches the nervous system, a peripheral administration is unlikely to be effective. Thus, there is a need to develop novel delivery methods for the siRNA swarms. However, in the case of human corneal HSV disease, the virus is most likely already a resident in the patient nerves and the treatment of local viral reactivations is of paramount importance.

In the peripherally limited infection (**Tables 10** and **11** and **Figures 12** and **13**), the UL29 swarm was able to inhibit viral replication and shedding significantly. The eGFP swarm had an effect at a point during the follow-up period. At the end of the study, the eyes and brains were measured for viral replication, of which there was none, in both wt and marker virus infected mice. TG explant culture showed no significant differences between different treatment groups (**Table 11**). In LoxLUC infected UL29-treated mice the amount of latently infected ganglia was slightly elevated but in the strain 17+ infected UL29-treated mice the latently infected ganglia were reduced. The peripheral dosing seemed to have little effect on latent virus.

All in all, UL29 swarm inhibited HSV-1 infection and alleviated symptoms in corneal HSV infection models. Moreover, UL29 protected the mice from a lethal corneal HSV challenge. Anti-HSV-UL29 swarm is a good lead for drug development against HSV, therefore to be developed further.

7 CONCLUSIONS

There is a need for new remedies against herpes simplex virus (HSV) which is a widespread pathogen. Despite the fact that the immune system of the host and available antivirals can inhibit the virus, there are situations where the viral disease takes over. In these cases, neither the immune system nor the drugs are capable of preventing damage caused by the infection. Such cases are common in immunocompromised patients and in HSV keratitis.

There are multiple approaches in antiviral drug development against HSV and there is a need for development of new treatment modalities against HSV. In addition, new and novel treatment methods, involving *tk* gene usage, of various diseases, such as cancer with HSV or HSV *tk* gene, can lead to situations where the usage of standard *tk*-based anti-HSV medication would be unwanted.

RNA interference (RNAi) is a promising tool in drug research. As a novel type of treatment, it is especially suitable for topical treatments. Standard, canonical, single site targeting small interfering (si)RNAs are susceptible to mutations in the viral genome, as a single nucleotide change could potentially render the virus resistant to the siRNA treatment. Moreover, the commercialization and patentability of widely used methods and very short nucleotide sequences could be difficult. These pose a problem to the potential drug development pipeline.

There is, however, a non-canonical way of producing siRNAs that are not susceptible to small mutations in the viral genome. Our enzymatically created novel siRNA swarms targeting large segments of target genes is a novel approach in antiviral drug research. Our studies show how anti-HSV-siRNA swarms are effective against both wild type and clinical field isolate strains of HSV. Moreover, a swarm targeting HSV gene *UL29* has proven wide efficacy, low innate immunity activation, shown no hint of antiviral resistance formation and has proven efficient in a corneal model as well. These results elucidate favorably the suitability of siRNA swarms in anti-HSV drug development.

In conclusion, the anti-HSV-siRNA swarm approach is feasible against HSV infection and harbours great potential for subsequent clinical implementation.

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