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To Nekku, Manda, and Malla

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a common virus of humans carried by half of the global population. After the primary infection, HSV has the ability to establish life-long latency, wherefrom it can reactivate. The latent state cannot be eliminated with modern pharmaceuticals, nor is there a vaccine available, despite massive efforts. Instead, the treatment focuses on diminishing viral replication. The current treatment, however, is insufficient, as it relies almost solely on acyclovir (ACV), and its derivatives, which share their mechanism of action, making ACV-resistant infections almost untreatable. Unfortunately, such infections are rather common, as severe HSV infections require long-term prophylactic treatment to prevent recurrences, which selects for ACV-resistant variants. The lack of treatment diversity against HSV-1 infections encourages for research on novel therapies.

Previously, enzymatically synthetized swarms of small interfering (si)RNA have been established as feasible means to treat HSV infection *in vitro* and *in vivo*. They differ from regular siRNA by their enzymatic synthesis and by their substantially longer target sequence. Thus, the emergence of resistance, even during long-term prophylactic treatment, is unlikely. However, as all RNA therapy, siRNA swarms face challenges with RNA stability. Therefore, in this study, the goal was to improve the siRNA swarms by synthesizing novel anti-HSV siRNA swarms with chemical 2′-fluoro-modifications to increase RNA efficacy and stability. The modified siRNA swarms, representing modifications of each nucleotide, were first validated *in vitro* in cells of the nervous system. The research was continued in a highly translational cell line representing the human cornea, which we first validated for use in antiviral RNAi studies. In both cell types, the modified siRNA swarm(s) proved well tolerated and potent beyond the unmodified counterparts, with only modest effects on the host innate responses, even in the presence of viral challenge. Furthermore, all studied HSV-1 strains, including various clinical isolates, were highly sensitive to both modified and unmodified siRNA swarms, whereas their ACV sensitivity varied, proving the potential of siRNA swarms for future therapeutic use.

This study shows that incorporation of modified nucleotides to the anti-HSV siRNA swarms is advantageous, and should therefore be preferred in future studies.

KEYWORDS: herpes simplex virus, antiviral, RNA interference, siRNA, acyclovir, innate immunity, drug research

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TIIVISTELMÄ

Herpes simplex -virus 1 (HSV-1) tunnetaan parhaiten yskänrokon aiheuttajana, mutta virus aiheuttaa myös vakavampia tautitiloja, kuten sokeuttavaa herpeskeratiittia. HSV-1 pystyy ensi-infektion jälkeen piiloutumaan ihmisen elimistöön latentissa muodossa, joka voi aktivoitua esimerkiksi stressin takia. Latenttia herpesinfektiota ei osata vielä parantaa, eikä HSV-rokotteen kehityksessä ole onnistuttu, ja siksi herpesinfektioiden lääkehoito keskittyy lähinnä akuuttien herpesepisodien hoitoon. Vaihtoehtoina lääkehoitoon ovat kuitenkin lähinnä vain asikloviiri ja sen johdannaiset, jotka ovat toimintamekanismiltaan identtisiä. Tämä on johtanut lääkeaineresistenttien infektioiden kehittymiseen esimerkiksi herpeskeratiittipotilailla, jotka tarvitsevat ennaltaehkäisevää hoitoa jopa pysyvää kudostuhoa aiheuttaviin uusiutuviin infektioihin. Resistentit infektiot voivat olla todella vaarallisia, sillä niitä on vaikea hoitaa, ja ne uusiutuvat helposti.

Entsymaattisesti tuotetut pieniä hiljentäviä RNA molekyylejä sisältävät parvet, eli siRNA-parvet ovat mahdollinen vastaus lääkeaineresistenttien HSV-infektioiden hoitotarpeeseen. siRNA-parvet eroavat tavallisista siRNA:sta niiden jopa tuhansien nukleotidien pituisella kohdesekvenssillä, joka vähentää resistenssin kehittymisen mahdollisuutta lääkehoidon aikana. HSV:en kohdistetut siRNA-parvet ovat jo aiemmin osoittaneet tehonsa niin solu- kuin eläinmalleillakin. Kuitenkin, kuten RNA-hoidolle on tavallista, RNA:n säilyvyys vaatii parannuksia. Siksi tässä väitöskirjassa tarkoituksena oli tutkia, mikäli siRNA-parvet joissa on stabiiliutta lisääviä 2′-fluoro-muunneltuja nukleotideja, voisivat olla vaihtoehto HSV-infektioiden hoitoon. Muunnellut siRNA-parvet validoitiin ensin hermoston soluissa, jonka jälkeen tutkimusta jatkettiin herpeskeratiittia mallintavassa sarveiskalvon epiteliaalisessa solulinjassa. Jokainen muunneltu siRNA-parvi oli molemmissa solulinjoissa hyvin siedetty sekä antiviraalisesti tehokkaampi, kuin tavalliset siRNA-parvet. Lisäksi kaikki tutkitut HSV-1 kannat, myös potilaista eristetyt ja asikloviiriherkkyydeltään muuttuneet kannat, olivat herkkiä siRNA-parville.

Tämän väitöskirjan tutkimus osoittaa, että muunneltujen nukleotidien käyttö siRNA parvissa on hyödyllistä, ainakin HSV-infektion hoidossa, ja että muunnellut siRNA-parvet voisivat olla erittäin lupaavia lääkeaineresistenttien HSV-infektioiden hoidossa.

AVAINSANAT: herpes simplex -virus, lääkekehitys, viruslääkehoito, asikloviiri

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Abbreviations

10% F-N	A modified siRNA swarm containing partially of a particular nucleotide as a 2′-fluoro-modified version. The modified nucleotide (N) can be either A (adenosine), C (cytidine), or U (uridine).
100% F-N	A modified siRNA swarm containing 100% of a particular nucleotide as a 2′-fluoro-modified version. The modified nucleotide (N) can be either A (adenosine), C (cytidine), or U (uridine).
17+	HSV-1 reference strain, HSV-1(17+)
88 bp	an 88 bp long dsRNA, used as a positive control for cytotoxicity
α-TIF	alpha trans-inducing factor of HSV, activator of transcription, also referred as VP16, encoded by UL48 gene
Δ305	a thymidine kinase deficient strain of HSV-1
ACV	acyclovir
ASO	antisense oligonucleotide
bp	base pair
CC_{50}	half-maximal cytotoxicity value
CNS	central nervous system
DdRp	DNA-dependent RNA polymerase
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dpi	days post infection
dsRNA	double stranded RNA
EMA	European Medicines Agency
EMEM	Eagle's minimum essential medium
F	HSV-1 reference strain, HSV-1 (F)
FBS	fetal bovine serum
FDA	U.S Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene
gB, gC, …	glycoprotein B, glycoprotein C, …
GFP	green fluorescent protein
hiFBS	heat inactivated FBS
HCE	human corneal epithelial cell line

HCMV	human cytomegalovirus
HHV	human herpesvirus
HIV-1	human immunodeficiency virus type 1
hpi	hours post infection
HPI	helicase-primase inhibitor
hpt	hours post transfection
HSE	herpes simplex encephalitis
HSK	herpes simplex keratitis
HSV-1	herpes simplex virus type 1
HSV-1-GFP	recombinant HSV-1 strain expressing GFP from a mouse cytomegalovirus promoter, HSV1(17+)LoxP$_{mCMV}$GFP
HSV-2	herpes simplex virus type 2
IC_{50}	half maximal inhibitory concentration
ICP	infected cell protein
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL-29	interleukin 29, also known as IFN-λ1
ISG	interferon stimulated gene
IND	investigative new drug application, an FDA application for a new investigative drug required to administer to humans
KOS	HSV-1 reference strain, HSV-1 (KOS)
LAT	latency associated transcript
MOA	mechanism of action
miRNA	micro RNA
mRNA	messenger RNA
MxA	myxovirus resistance protein 1
MxB	myxovirus resistance protein 2
nt	nucleotide
PCR	polymerase chain reaction
pfu	plaque forming unit
PKR	protein kinase R
PRR	pattern recognition receptor
qPCR	quantitative PCR
RdRp	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RT	room temperature
RT-qPCR	reverse transcriptase quantitative PCR
RNA	ribonucleic acid

RNAi	RNA interference
SADBE	squaric acid dibutylester
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2, the causative virus of the COVID-19 pandemic
SD	standard deviation
SEM	standard error of the mean
siRNA	small interfering RNA
ssRNA	single stranded RNA
TLR	toll-like receptor
TK	thymidine kinase
TG	trigeminal ganglion
U373MG	neuroglioma cell line, currently reclassified as U-251
U_L	unique sequence long segment of the HSV genome
UL23	unique sequence long segment gene 23, a β-gene encoding TK
UL29	unique sequence long segment gene 29, a β-gene encoding ICP8, a major DNA binding protein
UL30	unique sequence long segment gene 30, a β-gene encoding viral DNA polymerase
UL48	unique sequence long segment gene 48, a γ-gene encoding VP16/α-TIF
U_S	unique sequence short segment of the HSV genome
US1	unique sequence short segment gene 1, an α-gene encoding ICP22
vhs	virion host shutoff protein
VP	viral protein
VP16	see α-TIF
VZV	varizella-zoster virus

List of Original Publications

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

I Levanova A A[*], **Kalke K**[*], Lund L M, Sipari N, Sadeghi M, Nyman M C, Paavilainen H, Hukkanen V, and Poranen M M. Enzymatically synthesized 2'-fluoro-modified Dicer-substrate siRNA swarms against herpes simplex virus demonstrate enhanced antiviral efficacy and low cytotoxicity. *Antiviral Research*, 2020; 182: 104916. [*]Equal contribution

II **Kalke K**, Lehtinen J, Gnjatovic J, Lund L M, Nyman M C, Paavilainen H, Orpana J, Lasanen T, Frejborg F, Levanova A A, Vuorinen T, Poranen M M, and Hukkanen V. Herpes simplex virus type 1 clinical isolates respond to UL29-targeted siRNA swarm treatment independent of their acyclovir sensitivity. *Viruses*, 2020; 12: 1434.

III **Kalke K**, Lund L M, Nyman M C, Levanova A A, Urtti A, Poranen M M, Hukkanen V, and Paavilainen H. Swarms of chemically modified antiviral siRNA targeting herpes simplex virus infection in human corneal epithelial cells. *PLoS Pathogens*, 2022; 18: e1010688.

1 Introduction

Herpes simplex virus type 1 (HSV-1) is a life-long nuisance of approximately half of the global population. Although the virus is often associated with recurrent cold sores, it also causes severe diseases, such as the potentially blinding herpes keratitis. The severe diseases often require prophylactic treatment to prevent recurrences, which may cause dangerous exacerbations. However, when the currently available pharmaceuticals are used prophylactically for longer periods of time, they are known to cause emergence of drug-resistant strains, especially with immunocompromised patients. These strains are often resistant to majority of, if not all available treatment, as all available treatment targets the same viral protein. The lack of treatment diversity and of a vaccine lead to a need for novel antivirals against HSV infection.

The current clinical pipeline for treatment of HSV infection is unsurprising, ranging from monoclonal antibodies to small molecule inhibitors. Despite the evident potential, drug candidates which would utilize RNA interference (RNAi) towards viral transcripts are absent. With other viruses there have already been success stories even in early phase clinical trials. Small interfering RNAs (siRNAs) act via RNAi and could offer the much-needed novel mechanism of action for treatment of HSV. The diseases caused by HSV are mostly topical, and therefore the delivery of siRNA would not be as challenging as with systemic treatment, and the overall adverse effects could be limited.

In addition to delivery, siRNAs may be challenged by emerging resistance and by their stability. Previously, to circumvent any emerging resistance, antiviral siRNA swarms, which target multifold longer target sequences than regular siRNAs, have been established against HSV-1. The siRNA swarms have been well tolerated and efficient both *in vitro* and *in vivo*, even against patient-derived clinical isolates. Here, to increase the stability of the siRNA swarms, we incorporated 2′-fluoro-modified nucleotides to their sequence. In addition, the modifications should increase the silencing potency and ergo the antiviral potency of the siRNA.

This thesis focuses on the safety and efficacy of modified siRNA swarms in treatment of HSV-1. The goal is to show that modified siRNA swarms could be a feasible treatment modality for severe HSV infections, especially for those resistant to acyclovir.

2 Review of the Literature

2.1 Herpesviruses

The members of the *herpesviridae* family are more commonly known as herpesviruses. According to the Virus Taxonomy Release of 2021 of the International Committee on Taxonomy of Viruses (ICTV), there are 115 members of the herpesvirus family, of which nine are considered as human herpesviruses (HHV) based on their transmission from human to human. Herpesviruses are large viruses, consisting of a bilayer envelope, tegument, icosahedral capsid, and a double-stranded DNA genome (**Figure 1A**). HHVs, as all herpesviruses, are capable of establishing latent infection after primary infection, from which they can periodically reactivate (Cohen, 2020).

HHVs represent all three of the herpesvirus subfamilies, *alpha*-, *beta*-, and *gammaherpesvirinae* more commonly referred to as α-, β-, and γ-herpesviruses, respectively, which are grouped based on their nucleotide sequences and biological properties. HHVs can cause varying pathologic outcomes in humans, which tend to be more severe in immunocompromised individuals. All nine HHVs, their estimated seroprevalences and the most common diseases caused are listed in **Table 1**. In this thesis, herpes simplex virus type 1 (HSV-1), which belongs to the α-herpesviruses, is the virus of interest, and is the main focus of the following chapters. Other HHVs belonging to the α-herpesviruses are herpes simplex virus type 2 (HSV-2), and varizella-zoster virus (VZV). HSV-1 and HSV-2 are relatively similar, and mostly cause recurrent lesions in either the oral or genital epithelium, respectively. VZV is commonly known for causing chicken pox upon primary infection and shingles (herpes zoster) upon reactivation. Notably, VZV is the only HHV against which there is a vaccine available, and the vaccine has been part of the Finnish vaccination programme since 2017 (Leino and Puumalainen, 2018).

Table 1. Human herpesviruses.

	HHV	Common name	Common diseases or symptoms[1]	Example(s) of more severe disease[1]	An estimate of global seroprevalence[2]	Reference
Alpha-herpesviruses	1	Herpes simplex virus 1 (HSV-1)	Orofacial and genital lesions	Retinitis, keratitis, encephalitis, neonatal herpes	~70%	(Looker et al., 2015a)
	2	Herpes simplex virus 2 (HSV-2)	Genital lesions	Neonatal herpes, encephalitis	~10%	(Looker et al., 2015b)
	3	Varizella-zoster virus (VZV)	Chickenpox (primary infection) and shingles (reactivation)	Pneumonitis	>90%[3]	(Bollaerts et al., 2017)
Beta-herpesviruses	5	Cytomegalovirus (HCMV)	Often asymptomatic, light fever, mononucleosis	Congenital HCMV of neonates, pneumonitis	~80%	(Zuhair et al., 2019)
	6A	Human herpesvirus 6A (HHV-6A)	Roseola	Encephalitis	>95%[4]	(De Bolle et al., 2005)
	6B	Human herpesvirus 6B (HHV-6B)	Exanthema subitum (Roseola infantum), Fever	Encephalitis	>95%[4]	(De Bolle et al., 2005)
	7	Human herpesvirus 7 (HHV-7)	Exanthema subitum (Roseola infantum), Fever	Encephalitis	>95%	(Clark et al., 1993)
Gamma-herpesviruses	4	Epstein-Barr Virus (EBV)	Often asymptomatic, mononucleosis	B cell lymphoma	>90%	(Tzellos and Farrell, 2012)
	8	Kaposi's sarcoma-associated herpes virus (KSHV)	Fever, rash	Kaposi sarcoma	<5% – >50%	(Rohner et al., 2014)

[1]HHV infections commonly cause less severe symptoms, but may also cause more severe diseases, especially in recurrent infections or in immunocompromised individuals. Columns with more common and severe diseases are modified from (Cohen, 2020).

[2]Please note that the seroprevalence may depend on many factors, such as geographical location, gender, socioeconomical status, and age. Furthermore, the most relevant factors may vary for each virus. Therefore, the global estimate of seroprevalence is too abstract to describe any local prevalence, incidence, or disease burden, but allows for an overall image.

[3]The estimate is from Europe, prior to immunizations. National studies from around the globe also suggest seroprevalences of 90% or over for the adult population (e.g. (Kose et al., 2013, Amjadi et al., 2017)).

[4]HHV-6A and HHV-6B are lacking a clear distinction in epidemiologic studies, as the two viruses were classified as distinct viruses and not HHV-6 variants only ten years ago in 2012 (Ablashi et al., 2014).

2.2 Biology of herpes simplex virus type 1 (HSV-1)

2.2.1 Virion and genome

The HSV-1 virion consists of the envelope, outer tegument, inner tegument, icosahedral capsid, and the dsDNA genome, in that order (**Figure 1A**). The virion size is roughly 170-200 nm, if the surface glycoproteins of the envelope are not taken into account (Grünewald et al., 2003). The viral envelope is a lipid bilayer with multiple embedded viral glycoproteins, which mediate cellular entry as well as interaction between HSV and the host immune system. Moreover, they are important targets for adaptive immunity, and thereby also for vaccine development. The most abundant glycoprotein is glycoprotein B (gB), which, together with gD and the heterodimer formed by gH and gL, are essential for viral entry by membrane fusion (Campadelli-Fiume et al., 2007).

Under the envelope lie the inner and outer teguments, which harbor various viral proteins critical for viral lifecycle (Loret et al., 2008). Among these critical proteins are for example VP16/α-TIF (encoded by UL48) and ICP0 (α0), responsible for initiation and transactivation of transcription (Kristie and Roizman, 1987, Kalamvoki and Roizman, 2010, Pellett et al., 1985), respectively, and for evasion of various host innate responses (Xing et al., 2013, van Lint et al., 2010, Orzalli et al., 2012). Another tegument protein rather necessary to mention in this context is the the virion host shutoff (vhs) protein (UL41), which mediates (for example) degradation of host mRNAs in the absence of *de novo* viral gene expression (Fenwick and McMenamin, 1984, Kwong and Frenkel, 1989), therefore preparing the cell to focus on generation of novel virions. In addition to the viral proteins, the tegument is also known to harbor various host proteins as minor components (Loret et al., 2008), but their role remains unclear.

Inside the tegument lies the capsid, protecting the viral DNA. The capsid of HSV is an icosahedral structure consisting of pentons, hexons and triplexes, formed by VP5 (UL19), VP19c (UL38), VP23 (UL18), and VP26 (UL35) (Yuan et al., 2018). The capsid structure is supported by capsid-associated tegument complexes, comprising the capsid proteins and tegument proteins UL17, UL25, and VP1/2 (UL36) (Dai and Zhou, 2018). The capsid-associated tegument complex is likely involved in capsid transport, and is of high importance in the capability of HSV for long-range neuronal transport.

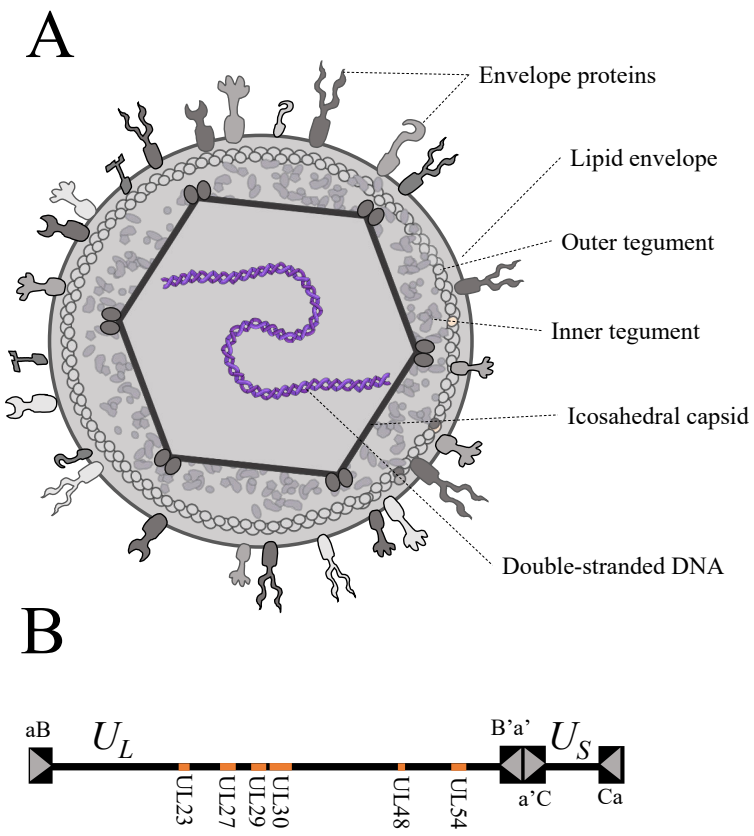


Figure 1. HSV-1 virion and genome. **(A)** HSV virion. The HSV virion consists of the envelope, outer tegument, inner tegument, icosahedral capsid, and a linear, double stranded DNA genome, which is approximately 152 kbps long. On the outer surface of the envelope are viral glycoproteins, guiding the first interactions between the virus and the host cell. **(B)** HSV genome. The HSV genome consists of repeat regions and unique regions, unique long (U_L) and unique short (U_S). The genes, which are either essential or nonessential, run in both strands, and may overlap. The U_L is flanked by repeat sequences b and b', and the U_S by repeat sequences c and c'. The b and c sequences are flanked by repeating a (or a') sequences, as according to the picture. Depending on the orientations of the unique sequences, the genome may be in one of four isomeric forms: As illustrated (prototype, P), with the unique long sequence inverted (I_L), with the unique short sequence inverted (I_S), or with both unique sequences inverted (I_{L+S}). Locations of few relevant genes for antiviral RNAi therapy are illustrated. The locations of the genes, as according to numbering of the JN555585.1 full genome sequence of HSV-1 17+ prototype strain are: UL23 (thymidine kinase) 37529–38659 (reverse strand); UL27 (gB) 43948–46662 (reverse strand); UL29 (ICP8) 49331–52921 (reverse strand); UL30 (DNA polymerase) 53633–57340 (forward strand); UL48 (VP16) 94400–95872 (reverse strand); UL54 (ICP27) 104529–106067 (forward strand). A and B modified from Kalke 2020.

The double-stranded, 152 kbp DNA genome of HSV is protected by the capsid. The genome is linear in the virion, but becomes circular upon nuclear entry, if there is no *de novo* viral protein synthesis (Strang and Stow, 2005, Garber et al., 1993). The genome contains more than 80 genes, which can be essential or nonessential for viral growth. Furthermore, the genes run on both genomic strands and can overlap. The genome has both unique and repeat sequences, which are illustrated in **Figure 1B**. The two unique sequences are referred as unique long (U_L) and unique short (U_S). Depending on the orientations of the unique sequences, the genome may be in one of four isomeric forms (Hayward et al., 1975). In the isomer chosen as the prototype (P), the genes of the unique sequences run in numerical order from left to right (UL1-UL56, US1-US12), as in the illustration in **Figure 1B**. The other three isomers have the unique long sequence inverted (I_L), the unique short sequence inverted (I_S), or both unique sequences inverted (I_{L+S}). Independent of the isomer, the U_L is flanked by inverted repeats referred as b and b', and the U_S is flanked by inverted repeats referred as c and c'. Both b and c genomic repeats code for genes, which are therefore in duplicate in the HSV genome. The b, b', c, and c' are flanked by a varying number (zero to many) of repeating a-sequences, which are highly conserved, but vary in length due to variable number of repeat elements within an a-sequence (Mocarski and Roizman, 1981, Mocarski and Roizman, 1982). Although the repeat sequences harbor genes and regulatory elements, and are essential in genome isomerization and DNA replication (Chou and Roizman, 1985, Chou and Roizman, 1986, Mocarski and Roizman, 1981, Deiss et al., 1986, Mocarski and Roizman, 1982), their role is perhaps not yet fully understood, as the currently published full genome sequences are determined via methods which cannot comprehensively distinguish the repeat sequences of HSV.

2.2.2 Lytic infection

As all viruses, HSV is not capable of producing progeny virions without harnessing a host cell machinery for the job. Therefore, to sustain its viral life cycle, HSV establishes lytic or latent infection in its host, humans. During lytic infection (**Figure 2A**) the host cell produces progeny virions according to the instructions of viral DNA and as guided by the viral proteins, which enter the cell for example in the tegument of the incoming virion. After successful egress of progeny virions, the host cell is usually destroyed – hence the name lytic infection. In clinical infection, the lytic infection is seen as growing lesions, whereas in cell culture, lytic infection is often detected as viral plaques (**Figure 2B**).

HSV, being an enveloped virus, enters the host cell by fusion with cellular membranes, which may also be endocytic (Sayers and Elliott, 2016, Nicola et al., 2005). First, the cell surface heparan sulphate proteoglycans (HSPG), are recognized

by gC. Then, gD interacts with, for example, nectin-1 or herpesvirus entry mediator (HVEM) to initiate fusion, which is subsequently executed by help of gB and the gH-gL heterodimer (Campadelli-Fiume et al., 2007). After successful entry, the capsid and tegument of the incoming virion are released to the cytoplasm. The capsid, protecting the viral DNA, is transported via microtubules and dyneins to the nuclear pores, wherefrom the viral DNA is released to the nucleus (Sodeik et al., 1997, Ye et al., 2000). As aforementioned, in addition to the viral DNA, important tegument proteins, such as ICP0 and VP16/α-TIF are translocated to the nucleus.

In the nucleus, the viral genes are expressed in a cascade-like manner (Honess and Roizman, 1974). The coordinately expressed gene groups are referred as α-, β-, and γ-genes, and their expression is initiated in that order, with the latter groups requiring expression of the prior. Some refer to the gene groups also as immediate early (IE), early (E), and late (L) genes, respectively, but such nomenclature may be confusing, as although cascade-like, the genomic groups are expressed simultaneously and the late -genes (γ-genes) should not be confused with latency associated genes or gene products. The α-gene transcription is initiated by the interaction of the viral VP16/α-TIF with a cellular transcription initiation complex consisting of HCF1, Oct1, and LSD1, among others (Kristie and Roizman, 1987, McKnight et al., 1987, Pellett et al., 1985). Subsequently, ICP0, translocated to the nucleus together with VP16/α-TIF, and being one of the first transcribed products as an α-gene, recruits and enhances CLOCK histone acetyl transferase activity, and therefore serves as a gene transcativator (Kalamvoki and Roizman, 2010). From here, the negative feed-back loop that is the cascade-like expression of the viral genes may initiate. In general, α-gene products are required for regulation of transcription whereas β-genes are required for replication of viral DNA, and γ-gene products are structural proteins, such as capsid proteins or glycoproteins.

The replication of the viral DNA (reviewed e.g. by Weller and Coen (2012), Bermek and Williams (2021)) is complex and not yet fully understood. Importantly, the process of DNA replication is highly conserved, as of the seven genes essential for DNA replication encoded by HSV (UL5, UL8, UL9, UL29, UL30, UL42, UL52) (Wu et al., 1988, Challberg, 1986, McGeoch et al., 1988b), at least six have homologues in other human herpesviruses as well (Bermek and Williams, 2021). The DNA replication initiates in one of the three viral origins of replication of the HSV genome: OriL (in U_L) or one of the two copies of OriS (in c and c'). The initiation requires the origin binding protein (OBP) (encoded by UL9) and the single-strand DNA binding protein ICP8 (encoded by UL29), which prepare (bind, unwind, and stabilize) the genome for replication (Weir et al., 1989, Weir and Stow, 1990, Boehmer and Lehman, 1993). Thereafter, the helicase-primase complex, consisting of products of UL52, UL5, and UL8, is recruited to unwind the DNA, which likely requires two or more copies of the complex (Chen et al., 2011). The DNA

polymerase (UL30) and its processive subunit (UL42) are recruited as the last pieces of the replication fork (illustrated in **Figure 2C**). Concatemeric DNA, required for DNA encapsidation, is suggested to be formed after circularization of the viral genome by rolling cycle replication, but the precise mechanism remains controversial (Weller and Coen, 2012, Bermek and Williams, 2021).

The viral DNA is packed in the nucleus into capsids to form nucleocapsids. On the way out of the nucleus, at the nuclear membrane, the nucleocapsid is primarily enveloped and thereafter de-enveloped, tegumented, and secondarily enveloped at vesicles of non-nuclear origin (Owen et al., 2015). The mature virions exit the cell via exocytosis, after which they may proceed to infect other, usually neighboring cells of the host, proceed to establish latent infection, or proceed to transmit to the next host (or another tissue of the same host) via secretions, such as saliva.

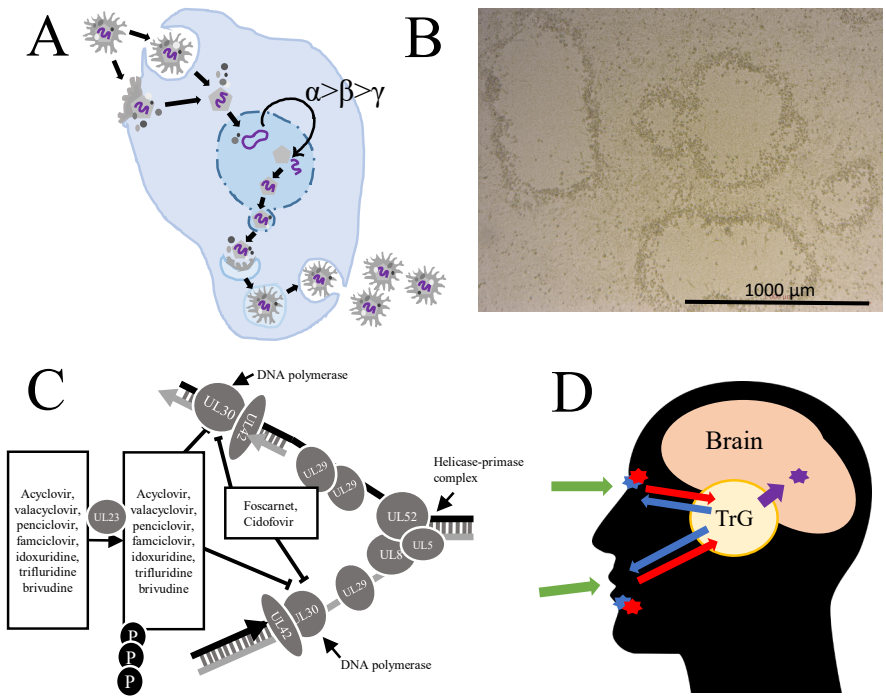


Figure 2. Lifecycle of HSV-1. **(A)** Lytic infection. The virus enters the host cell via fusion to cellular membranes. In the cell, the tegument proteins and capsid are released, and subsequently, the capsid is transported by nuclear pores, through which the viral DNA together with selected tegument proteins are translocated to the nucleus. In the nucleus, the viral genes are expressed in a cascade-like manner, from α- to β- to γ-genes, and the viral genome is replicated as illustrated in (C). The nucleocapsid is formed in the nucleus, and primarily enveloped already at the nuclear membrane, but rapidly de-

enveloped, tegumented, and secondarily enveloped at vesicles of non-nuclear origin. The mature virions exit the cells via exocytosis. **(B)** Viral plaques on Vero cells. Lytic infection in human cell cultures usually leads to formation of viral plaques. One plaque is formed by one plaque forming unit, from which the lytic infection proceeds to spread in the cell culture as seen by growth of the original plaque, and by formation of secondary plaques, formed by progeny viruses spreading via the culture medium. The HSV-1 causing plaque formation in Vero cells shown in this figure is derived from a patient. **(C)** DNA replication fork and current antiviral treatment. The replication fork is a simplified visualization on how the viral DNA replicates based on the reviews by Weller and Coen (2012), and Bermek and Williams (2021). In short, the helicase primase complex, consisting of UL5, UL8, and UL52 gene products, unwinds the genome. The mechanisms of action of all currently available treatments of HSV are shown in the picture. The majority require activation by phosphorylation by the viral thymidine kinase (UL23), but in principle, all available treatments target the DNA polymerase (UL30). **(D)** From (primary) lytic infection to latent infection. After transmission (green arrows) via e.g. saliva, a primary lytic infection (red star), which may be symptomatic or asymptomatic, takes place. The most common site for primary infection is the epithelium in the oro-labial area. After a primary infection, the virus can establish latency in the somas of the trigeminal ganglia (TG). The virus transports to the TG from the epithelium via retrograde transport (red arrows) in the axons of the sensory neurons. From the TG, upon reactivation, the virus may proceed back to the epithelium via anterograde transport (blue arrows) in the axons of the sensory neurons, and re-establish a lytic infection (blue stars), which may again be asymptomatic or symptomatic. It is more likely for the lytic infection to reoccur in the original site of infection upon reactivation, but it may also travel to other epithelial sites via the trigeminal nerve branches. In unfortunate scenarios, the virus may proceed to infect the brain (purple arrow), causing potentially fatal lytic infection in the brain, referred to as HSV encephalitis. Though the illustration is of the cephalic area, it is important to remember that HSV-1 is an important cause of herpes also in the genital area, whereby the latency is established in the sacral dorsal root sensory ganglia. Figures 2A and 2D are from Kalke (2020) and Figure 2C is modified from Frejborg et al. (2022).

2.2.3 Latent infection

HSV virions may proceed to establish latent infection (**Figure 2D**). During latent infection, the virus hides from the host, and is basically undetectable until reactivation. To establish latency, the virions need to transport to somas of sensory neurons via their axons. The sensory somas are bundled as sensory ganglia, such as the trigeminal ganglia or the sacral dorsal root sensory ganglia, which are the most common ganglia for HSV latency following infection in cranial or genital areas, respectively. The establishment and upkeep of latency are rather complex, but comprehensive reviews, such as Nicoll et al. (2012) and Cohen (2020), exist. In short, during the latent state, the HSV genome is in an episomal form in the somas, and only a few RNAs, namely the latency associated transcript (LAT) and a handful of micro RNAs (miRNA) are expressed (Stevens et al., 1987, Umbach et al., 2008, Cokarić Brdovčak et al., 2018). Simplified, the role of LAT and the miRNAs is to inhibit apoptosis of the host cell and to prevent expression of other viral genes, such as ICP0, to prevent initiation of transcription of viral genes, and thus reactivation. The exact mechanism of HSV reactivation is not thoroughly understood, but factors

associated with reactivation are for example ultraviolet radiation, stress, fever, or trauma to the nerves. During reactivation from latent infection, α-, β-, and γ-genes are simultaneously expressed in the first wave, and only thereafter cascade-like, as during lytic infection (Mattila et al., 2015, Linderman et al., 2017).

2.2.4 Immune evasion

The incoming virion is recognized by the cell by various pattern recognition receptors (PRR). The PRRs can detect pathogen or damage associated molecular patterns (PAMPs or DAMPs, respectively), such as foreign nucleic acids and viral proteins. The recognition of the virion enables the cell to put up innate antiviral defenses, and alert neighboring cells or tissues of incoming foreign material. Among the most important innate antiviral responses are interferons, which are highly antiviral and even used as medicine to treat viral infections (Isaacs and Lindenmann, 1957, Minkovitz and Pepose, 1995). The outcome of the clinical infection highly depends on the capability of the host to sustain high enough, yet not overly extensive, innate and adaptive antiviral responses. Therefore, individuals with inborn errors in innate antiviral responses are usually at larger risk of developing severe disease in response to viral infection (Zhang et al., 2007).

Although cells do put up effective walls to prevent viral infections, HSV is capable of circumventing both innate and adaptive antiviral responses and thus establish functional viral replication also in cells of healthy individuals. Already upon entering the cell, toll-like receptor (TLR) 2 recognizes viral surface glycoproteins and initiates production of proinflammatory cytokines (Kurt-Jones et al., 2004). The activity of TLR2 is, however, inhibited by the viral ICP0 (van Lint et al., 2010), which is delivered to the cell in the tegument. The viral DNA, whether incoming or synthesized, is recognized by multiple PRRs, including TLR9, cGAS (cyclic GMP-AMP synthase), and IFI16 (gamma-interferon-inducible protein 16), which all induce production of type I interferons. Of these DNA recognizing PRRs, at least IFI16 is directly affected by viral proteins, as it is degraded by ICP0 (Orzalli et al., 2012). DsRNA, produced during viral replication, is recognized by at least TLR3 and PKR (protein kinase R), which in turn are directly inhibited by US3 (Peri et al., 2008) and ICP34.5 (He et al., 1997), respectively. Single site (ss)RNAs, also produced during viral replication, are recognized by at least TLR7, TLR8 and RIG-I. Activation of TLR7, TLR8, and RIG-I, as well as of TLR3 and PKR, lead to induction of the type I interferon pathway.

The type I interferon pathway induces expression of antiviral type I interferons (such as IFN-α, IFN-β, IFN-κ), which subsequently induce production of interferon stimulated genes (ISGs). The interferon pathway, which may be mediated via various cellular proteins, is also inhibited by various viral proteins, such as ICP27 (UL54),

VP16, ICP34.5 and vhs (Xing et al., 2013, Verpooten et al., 2009, Christensen et al., 2016). Examples of ISGs are human myxovirus resistance protein 1 and 2 (MxA, MxB, respectively). MxA and MxB reduce the replication potency of HSV-1 and inhibit the delivery of the viral genome to the nucleus, respectively, yet their activity is modified by HSV-1 to support viral replication (Ku et al., 2011, Crameri et al., 2018). Notably, HSV also inhibits PKR mediated autophagy via ICP34.5 (Orvedahl et al., 2007) and transcription of host mRNA via vhs (Kwong and Frenkel, 1989, Kwong et al., 1988, Read and Frenkel, 1983), thus allowing a stable virus-focused environment for generation of progeny viruses.

HSV is also capable of evading adaptive immune responses, which is clearly but indirectly proven by the lack of vaccine, despite massive efforts. HSV escapes the adaptive immune system at least by preventing complement activation (Friedman et al., 1984), by binding IgG (Dubin et al., 1991, Johnson et al., 1988), and by inhibition of both class I and class II major histocompatibility complexes (MHC), thus preventing presentation of viral antigens (Früh et al., 1995, Hill et al., 1995, Neumann et al., 2003).

As the ways of HSV to evade the immune responses of the host are many, the clinical outcome of the infection is always a balance resulting from many interactions. During antiviral treatment, the balance between the cell and the virus is interfered, as the cell will have a response to the incoming or prevailing treatment as well. The response to the treatment might of course be neutral or beneficial considering treatment outcome. However, the response to treatment may also result in an adverse inflammatory response, sensitizing the host to viral recurrences or to rapid infection spread. Therefore, understanding and studying the responses to antiviral treatment already at early stages of drug research and development is crucial.

2.3 HSV-1 is a pathogen

2.3.1 Diseases caused by HSV-1

HSV-1 is a common pathogen with no vaccine available to date and a global seroprevalence of approximately 70% (Looker et al., 2015a). The infection can be subclinical or symptomatic, both of which can result in viral shedding and thus also transmission (Wald et al., 1995, Barton et al., 1987, Mäki et al., 2015, Mäki et al., 2018). In Finland the seroprevalence of HSV-1 is below the global average, at roughly 50%, and like in other developed countries, has been decreasing for the past few decades (Puhakka et al., 2016, Kortekangas-Savolainen et al., 2014). The decrease in seroprevalence risks primary infection at a later age, which might increase the risk of developing for example neonatal herpes during pregnancy.

However, luckily, the incidence has remained unchanged thus far (Puhakka et al., 2016).

The common symptoms caused by HSV-1 are recurrent lesions (blisters, ulcers, "cold sores"), mainly in the oro-labial area, which can last for a few days or a few weeks. The first clinical signs of recurrent lesions are itching or tingling, which are rather familiar sensations to those living with recurrent herpes infection. Currently, genital lesions may also be considered as a common symptom of HSV-1 infection: in developed countries, such as in Finland, HSV-1 has surpassed HSV-2 as the most common cause of genital herpes among young women (Tuokko et al., 2014). The physical symptoms of genital herpes (lesions and related pain, fever, swollen lymph nodes) are most severe upon primary infection, whereas possible recurrences tend to be milder and decrease in frequency over time (WHO, 2017). Additionally, genital HSV-1 may protect from HSV-2 infection (Xu et al., 2002).

2.3.1.1 Neonatal herpes

Neonatal herpes, a dangerous complication of genital herpes, is a complication of roughly 1000 childbirths annually in Europe (Looker et al., 2017). Reflecting the increasing proportions of HSV-1 as the causative agent of genital herpes, HSV-1 is the main causative pathogen (57% of cases) of neonatal herpes in Europe (Looker et al., 2017). In neonatal herpes, HSV transmits from the mother to the infant, most commonly during childbirth. The transmission may result from either recurrent or primary infection, but primary infections pose the highest risk (Brown et al., 2003). The symptoms vary, but a bit less than half of the infants develop potentially fatal CNS symptoms, commonly with subsequent neurodevelopmental outcomes (James and Kimberlin, 2015). Additionally, neonatal herpes always has a risk of fatal disseminated infection, but the risk is most severe in the more rare cases, where the infection takes place already *in utero* (James and Kimberlin, 2015).

2.3.1.2 Herpes simplex encephalitis

Another severe disease caused by HSV-1 is herpes encephalitis (HSE), the most common cause of sporadic, infectious encephalitis. HSE is rather rare, with 1500-3500 cases in Europe per year (Whitley, 2006), but is very severe even when treated with the best know-how. In fact, with intravenous acyclovir (ACV) treatment, which is the preferred treatment, HSE has a mortality rate of 15-20%, whereas without treatment the mortality rate is 50% (Raschilas et al., 2002, Välimaa et al., 2013). The symptoms include for example weakening, convulsions, paralysis and cognitive disturbances. HSE develops when HSV-1, during primary or recurrent infection, enters the brain and establishes lytic infection (**Figure 2B**). The adverse access of

HSV-1 to the brain can be a consequence of overall high viral load, increased neurotropism of a (re)activated viral strain, or lacking antiviral defenses of the patient, such as TLR3 deficiency (Zhang et al., 2007). However, notably, HSE appears as likely to develop in immunocompromised as in immunocompetent individuals (Whitley, 2006). The possible routes for HSV-1 to enter the brain include at least the trigeminal pathway (as in **Figure 2B**), the olfactory nerve routes, and hematogenous spread of infection.

2.3.1.3 Herpes simplex keratitis

HSV-1 infection on the epithelium or stroma of the cornea is referred as herpes simplex keratitis (HSK). HSK is the most common cause of infectious blindness in the developed countries (Dawson and Togni, 1976), where it has an estimated incidence of 250,000 cases per year with 1.5% of cases leading to severe visual impairments, including blindness (Farooq and Shukla, 2012, Labetoulle et al., 2005). Extrapolated, this would mean approximately 140,000 cases per year in Europe, with roughly 2,000 annual cases of severe visual impairment. HSV-1 infection of the cornea may result from primary infection of the eye, or a reactivation of an oro-labial infection to the cornea (**Figure 2B**), which would most likely take place on the ipsilateral side (Labetoulle et al., 2003, Labetoulle et al., 2000). After the first corneal infection, which is commonly not severe, recurrences in the cornea are likely, adding to disease severity with growing lesions, increasing inflammation, sensation loss, and inflammation of surrounding tissues (Dawson and Togni, 1976). Eventually, the responses to the recurrent infections can trigger an (overly) extensive immune response, which may result in destruction of the corneal stroma and subsequent severe visual impairment. For this reason, HSK patients require long term ACV prophylaxis, which adversely predisposes the patient for emergence of ACV-resistant infection (van Velzen et al., 2013, Duan et al., 2009, Duan et al., 2008).

2.3.2 Current treatment

Due to the currently undruggable latent infection, HSV-1 infection cannot be cured, per se. Therefore, the current treatment of HSV-1 is focused on treating lesions, or preventing recurrences by prophylactic treatment. In general, the first-in-line (prophylactic and therapeutic) treatment of HSV-1 infection is a guanosine analogue, ACV, or one of its derivative compounds (Koulu and Mervaala, 2013). The mechanism of action (MOA) of ACV is based on the activity of viral thymidine kinase (TK) (encoded by UL23) and viral DNA polymerase (UL30). In infected cells, TK phosphorylates ACV to its monophosphate form, from where the cellular kinases phosphorylate it to its active triphosphate form. Triphosphate-ACV

competitively inhibits the activity of viral DNA polymerase, and prevents further synthesis of the viral DNA chain (**Figure 2C**). The MOA requiring TK activity ensures treatment safety, as the biologically active substance is only present in infected cells. The MOA of ACV is shared by other guanosine analogues, including the ACV prodrug with increased oral bioavailability, valacyclovir, as well as penciclovir and its prodrug famciclovir, as well as by 2′-deoxyuridine analogues idoxuridine, trifluridine, and brivudine. These aforementioned drugs are highly efficient and well tolerated in use, and have topical, oral, and intravenous options available as well. However, viral strains may be, or may develop to be resistant to ACV (e.g. Bergmann et al., 2017, Burns et al., 1982, Duan et al., 2008, Darville et al., 1998, Parris and Harrington, 1982, Schnipper et al., 1982). In such occasions, usually, also other drugs requiring activation by TK are redundant, and other options for treatment are required. Basically, the only feasible option in such scenario is foscarnet (intravenous), which also competitively inhibits the viral DNA polymerase, but is non-TK-dependent (Bergmann et al., 2017, Sauerbrei et al., 2011). Foscarnet is, however, not well tolerated as it has high renal toxicity, and other severe adverse effects. Were there resistance to foscarnet as well, or contraindications to its use, an even more toxic drug, cidofovir (intravenous), may be considered for treatment (Blot et al., 2000). Cidofovir, normally used to treat HCMV, targets the activity of the viral DNA polymerase and is non-TK-dependent.

2.3.3 Diversity of clinical isolates and emerging resistance to current treatment

Clinical, circulating strains of HSV-1 can vary not only by their genotype (commonly 1-3% nucleotide variation), but also by their phenotype (Bowen et al., 2019, Kalke et al., 2022). Usually, the genotype does not predict the phenotype, and therefore, the viral phenotype has not yet been connected to any genomic groups nor any pattern of geographic diversity (Bowen et al., 2019, Shipley et al., 2018, Kalke et al., 2022). In addition to abundant interstrain variation, also intrastrain variation exists, meaning that within a clinical isolate, or even a prototype laboratory strain, there are many viral strains with differing geno- and phenotypes present (Parsons et al., 2015). The intrastrain variation of HSV-1 enables the virus to adjust to prevailing circumstances via survival of the fittest during the viral lifecycle. A very important consequence of intrastrain variation is the possibility for emergence of treatment resistant strains, as an antiviral can adversely select for a variant from the pool of viruses which best tolerates its presence. Subsequently, fully treatment resistant strains, such as ACV-resistant strains, may emerge. In such cases, and as ACV shares its mechanism of action basically with all available treatment, the ACV-resistant strains may lead to recurrences or exacerbations, which cannot be cured using any

available treatment. Such strains can be resistant even to foscarnet, despite its non-TK-dependence (Duan et al., 2008, Danve-Szatanek et al., 2004). For this reason, ACV-resistant HSV infection is currently considered an unmet medical need.

ACV-resistant strains usually have a mutation in their TK gene (UL23), leading to altered or deficient TK activity (Schmidt et al., 2015, Kimberlin et al., 1995). Altered or deficient TK activity prevents the phosphorylation of ACV and related compounds to their active phosphorylated form, which is required by almost all available treatment (**Figure 2C**). TK (UL23) is a nonessential gene, and TK can therefore be considered prone to harbor mutations altering its activity, as the proper function of TK is nonessential for viral replication. Therefore, rather unsurprisingly, there is prevalent variability in the ACV sensitivity of clinical HSV-1 isolates (Pan et al., 2014, Bowen et al., 2019, Schmidt et al., 2015, Kalke et al., 2022, McLaren et al., 1982). Mutations in DNA polymerase (UL30) can also cause decreased sensitivity or resistance to ACV, and also to other available treatment (**Figure 2C**). Basically, all available treatment functions via inhibition of the activity of the DNA polymerase and in principle, an HSV strain with an altered DNA polymerase, is resistant to all available treatment. Fortunately, such mutations are rarer than those in TK (UL23), as the DNA polymerase gene UL30 is essential, and therefore strains with UL30 variations altering its activity, or active site, are not as likely to be fit (Schmidt et al., 2015). Nevertheless, alarmingly, UL23 and UL30 mutated ACV-resistant strains exist, and both of the genes harbor high genetic variability among HSV-1 strains (Sauerbrei et al., 2011, Schmidt et al., 2015).

ACV-resistant strains were first described in immunocompromised individuals (Schnipper et al., 1982, Sibrack et al., 1982, Burns et al., 1982, Parris and Harrington, 1982), but have been shown to emerge also in immunocompetent patients (Duan et al., 2009, Duan et al., 2008, van Velzen et al., 2013, Pan et al., 2014, Bergmann et al., 2017). According to estimations, 0.1-0.7% of all infections of immunocompetent individuals are ACV-resistant (Farooq and Shukla, 2012, Christophers et al., 1998). The ACV resistance becomes more common when the patient needs long term prophylactic treatment (van Velzen et al., 2013). Such treatment is required in many of the more severe diseases, for example in herpes keratitis, to prevent recurrences and subsequent exacerbations. For example, in a study of herpes keratitis patients up to 6.4% of immunocompetent patients were suffering from ACV-resistant infection (Duan et al., 2008). The prevalence is even higher in immunocompromised patients. Depending on the estimation, 3-10% of immunocompromised patients suffer from an ACV-resistant infection (Danve-Szatanek et al., 2004, Stranska et al., 2005). However, in allogenic bone marrow transplant patients, who commonly receive prophylactic ACV, the number of ACV-resistant infections has been reported as 7-25% (Danve-Szatanek et al., 2004, Morfin et al., 2004, Chakrabarti et al., 2000). Alarmingly, up to half of the bone marrow transplant patients reported to have an

ACV-resistant infection, were also resistant to foscarnet (Danve-Szatanek et al., 2004), which is used to treat ACV-resistant infection. Simultaneous resistance to ACV and foscarnet is reported also elsewhere, even in immunocompetent individuals (Darville et al., 1998, Duan et al., 2008, Chakrabarti et al., 2000, Blot et al., 2000). Likely, such strains harbor protein activity altering mutations in UL30, or in UL23 and UL30 simultaneously.

2.3.4 Clinical pipeline for novel anti-HSV-1 medicines

ACV resistance, and its rather common emergence during prophylactic, long term treatment, combined with its lacking safe treatment options, and high mortality in HSE, despite treatment, have created an unmet medical need for an antiviral having a novel mechanism of action in contrast to the available treatment. In **Table 2**, the current clinical pipeline for antiviral medication for HSV-1 infection, as available in the clinicaltrials.gov database, is summarized. Altogether, the pipeline has a total of 12 investigative drugs, of which a majority is focusing on treatment of orolabial lesions, and only few to the more severe diseases. Furthermore, only half of the pipeline may be considered as HSV-specific treatment, whereas the rest are e.g. immunomodulatory treatments. The three groups representing HSV-specific treatment in the current antiviral pipeline are biopharmaceuticals, small molecule inhibitors, and gene editing drugs.

Small molecule inhibitors in the pipeline are helicase-primase inhibitors (HPIs), pritelivir (AIC316) and amenamevir (ASP2151) (**Table 2**), which represent a promising novel MOA for anti-HSV medicines. The HPIs target the helicase-primase complex, which has an essential role in HSV DNA replication (**Figure 2C**). Pritelivir was successful in its phase II trial against HSV-2 in comparison to valacyclovir, and showed no emerging resistance when used daily for four consecutive weeks (Edlefsen et al., 2016, Wald et al., 2016). The phase III study, targeting ACV-resistant mucutanous infection of immunocompromised individuals, which is currently recruiting, does not clearly differentiate between HSV-1 and HSV-2. Amenamevir, which is already approved in Japan for use against VZV under the trade name Amenalief®, has completed its phase III studies in Japan. Subsequently, the company has filed for extension for Amenalief® with an indication to recurrent HSV infection in Japan. Although the novel MOA of the HPIs is promising, HSV variants resistant to pritelivir or amenamevir have already been reported (Biswas et al., 2007a, 2007b, Chono et al., 2012). The mutations leading to HPI resistance have been mainly associated with UL5 (Chono et al., 2012, Biswas et al., 2009, 2008). The resistance enabling mutations have usually attenuated the strains, but also resistant strains without any abnormalities in virulence or fitness have been discovered (Sato et al., 2021).

Table 2. Pipeline for antiviral drug development against HSV-1, as of 7/2022 and as in Clinicaltrials.gov. Drug candidates with trials initiated after year 2010 are included. Table modified from Kalke (2020) and from Frejborg et al. (2022).

Investigative drug	Indication	Route of administration	Treatment approach	Phase and status		Clinical trials.gov identifier
Small molecule ihibitors (HSV-specific)						
Amenamevir (ASP2151)	Orolabial, facial or genital HSV lesions	Oral	Inhibition of viral helicase-primase complex	III	Completed[1]	NCT01959295
Pritelivir (AIC316)	Acyclovir-resistant mucocutaneous HSV (immunocompromised patients)	Oral		III	Recruiting	NCT03073967
Brincidofovir (CMX001)	Neonatal herpes with CNS disease	Oral	Prodrug of cidofovir	I/II	Withdrawn	NCT01610765
Gene editing (HSV-specific)						
Gene editing therapy (BD111)	Herpes keratitis	Corneal injection	CRISPR/Cas9 mRNA	I/II	Completed[2]	NCT04560790
Biopharmaceuticals (HSV-specific)						
Monoclonal gB-antibody (HDIT101)	Recurrent orolabial HSV lesions	Topical	Monoclonal gB–antibody	II	Recruiting	NCT04539483
Monoclonal gD-antibody (UB-621)	HSV infection	Subcutaneous	Monoclonal gD-antibody	I	Completed[3]	NCT02346760
Immunomodulatory						
Squaric acid dibutyl ester (SADBE)	Prevention of recurrent orolabial HSV lesions	Topical	Immunosensitizer	II	Completed[4]	NCT02965781
Dexamethasone (corticosteroid)	Herpes simplex encephalitis	Intravenous	Anti-inflammatory, adjuvant for standard care	III	Recruiting	NCT03084783
Diclofenac, lidocaine (RMN3001)	Pain related to orolabial HSV lesions	Topical	Anti-inflammatory	II	Completed[3]	NCT02207881
Other						
Cryopreserved amniotic membrane (Prokera slim)	Herpes keratitis (dendritic)	Topical	Anti-inflammatory, anti-scarring and antiangiogenic	NA	Recruiting	NCT04598282
Photodynamic therapy	Orolabial HSV lesions	Topical	Photodynamic therapy	NA	Not yet recruiting	NCT04037475
NB-001	Orolabial HSV lesions	Topical	Disruption of the viral envelope	III	Active[5]	NCT01695187

[1]The company has filed for indication of amenamevir for recurrent herpes simplex infection in Japan [https://www.maruho.co.jp/english/information/20211214.html]

[2]Early results in China indicate success and the company is seeking investigative new drug (IND) -approval in U.S. (HerpesCureAdvocacy.com, 2022)

[3]Results unknown, or no results posted

[4]Seeking funding for phase III (HerpesCureAdvocacy.com, 2022)

[5]Company name (NanoBio Corporation) changed to BlueWillow, and NB-001 is not in their pipeline [https://bluewillow.com/vaccine-pipeline/]

Gene editing is represented by one investigative drug in the pipeline (**Table 2**), BD111, which is based on CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein 9) technology, whereby mRNA-carrying lentiviral particles deliver mRNA of Cas9, which breaks the viral gene(s) according to instructions by the co-delivered targeting RNA. BD111 is targeted to UL8 and UL29, which was highly efficient in an animal model of stromal herpes keratitis (Yin et al., 2021). The phase I/II trial in China has reported promising preliminary results, and is aiming to continue the clinical research in the U.S. (HerpesCureAdvocacy.com, 2022).

Biopharmaceuticals are represented in the pipeline by two early phase monoclonal antibodies, UB261 against gD, and HDIT101 against gB (**Table 2**). Both antibodies are HSV type-common and also studied in clinical trials against HSV-2. Both monoclonal antibodies function by neutralizing the virus and therefore preventing attachment to cellular surface. The MOA of monoclonal antibodies is however not necessarily absolute for treatment of HSV, as cell-to-cell spread, preferred by HSV, enables the virus to escape serum neutralization (Criscuolo et al., 2019).

Immunomodulatory therapy may increase in importance as an adjuvant in treatment of herpes infection. For example the use of corticosteroids has been suggested to be efficient as adjuvant therapy in herpes diseases (Arain et al., 2015, Wilhelmus et al., 1994). Indeed, corticosteroids, and other anti-inflammatory therapy, would enable prevention of exaggerated inflammation, which is a major cause of tissue destruction in (recurrent) severe herpes infection, such as in HSK and HSE. Modulation of immune responses, however, can also be seen as controversial, as disruption of the natural balance and natural antiviral defenses could also lead to adverse events (Lizarraga et al., 2013, Ramos-Estebanez et al., 2014). Nevertheless, the immunomodulatory treatments in the pipeline (**Table 2**) have had success in their trials. For example, SADBE, an immune system enhancer, has been reported to prevent recurrences better than placebo treatment (McTavish et al., 2019), and is currently searching for funding for phase III studies after a successful phase II.

None of the drugs in the pipeline, or any of the available treatment, can cure HSV infection, but only potentially treat, prevent, or reduce the active lesions. To cure the infection, eradication of the latent virus, or prevention of infection altogether with a prophylactic vaccine would be required. Targeting the very silent latent infection is considered a major challenge. However, recently, meganucleases, delivered by adeno-associated virus (AAV), were shown capable of eliminating a majority of the latent virus in an *in vivo* model of HSV infection (Aubert et al., 2020). Additionally, a few *in vitro* studies have shown promise by CRISPR/Cas9 mediated cleavage of episomal latent DNA (Wang and Quake, 2014), or with epigenetic modulation of

histones to prevent reactivation using histone demethylases (Hill et al., 2014, Liang et al., 2013) or methyltrasferases (Arbuckle et al., 2017).

2.3.4.1 A glimpse on the HSV vaccine pipeline

Development of a prophylactic vaccine against HSV-1 is an attractive choice to meet the unmet medical need. Previously, most vaccine development has focused on HSV-2 due to the global burden of genital herpes and its comorbidities. However, currently, the need for a vaccine preventing both HSV-1 and HSV-2 is more recognized, as HSV-1 has begun to take a leading role as the causative pathogen for genital herpes. Nevertheless, the capability of HSV to evade also adaptive immunity has challenged the vaccine development, which has had no success stories thus far, despite massive efforts. The massive efforts are represented by a total of three phase III studies, which all failed to meet their primary endpoints (Stanberry et al., 2002, Corey et al., 1999, Belshe et al., 2012). The vaccine candidates used in the trials were targeted to viral entry glycoproteins of HSV-2, either gD/gB or gD, to be more specific. Altogether, the vaccine pipeline with its successes and fails is not possible to review with well-earned attention in the scope of this thesis, but substantial, recent reviews on vaccine development are available (Awasthi and Friedman, 2022, Egan et al., 2020a), and a comprehensive pipeline of prophylactic vaccines currently in development is being collected online (HerpesCureAdvocacy.com, 2022). Altogether, the pipeline of prophylactic vaccines is in very early phases (preclinical or soon starting phase I), and in contrast to the previously failed glycoprotein subunit vaccines, basically all vaccine candidates are either live attenuated viruses or mRNA vaccines (HerpesCureAdvocacy.com, 2022). An example of a live attenuated virus is a gD deficient HSV-2 (ΔgD-2) by a company called X-Vax. The vaccine candidate has had promising preclinical results against both HSV-1 and HSV-2 (Burn et al., 2018, Visciano et al., 2021, Ramsey et al., 2020), based on which X-Vax has an Investigative New Drug (IND) –application in progress for the experimental vaccine against herpes simplex virus 1 and 2 (HSV-1 and -2), aiming to start clinical phases in 2024. Another highlight in the pipeline are mRNA vaccines, to which the successes in the SARS-CoV-2 pandemic induced vaccine race have paved the regulatory way. Two companies, BioNTech and Moderna, are bringing their mRNA vaccine candidates to the pipeline, with Moderna's candidate (mRNA-1608) still in preclinical phase, with reported potential cross-protection against HSV-1, and BioNTech mRNA vaccine targeting both HSV-1 and HSV-2 potentially starting phase I already in late 2022. The mRNA vaccine by BioNTech, developed in collaboration with Dr. Friedman laboratory (University of Pennsylvania), is a trivalent mRNA vaccine containing coding sequences for gD, gE, and gC of HSV-2, as gC is a major antigen of HSV (Adamiak et al., 2010). This vaccine has been shown

to protect from both HSV-1 and HSV-2 in *in vivo* studies (Egan et al., 2020b, Awasthi et al., 2019).

2.4 Antiviral small interfering RNAs

2.4.1 RNA interference

RNA interference (RNAi) is a conserved cellular mechanism, which leads to sequence specific destruction of RNA molecules in eukaryotes (Cerutti and Casas-Mollano, 2006). RNAi is mediated by many species of endogenous small noncoding RNA molecules, including short interfering RNAs (siRNAs) and micro RNAs (miRNAs), which are somewhat overlapping in function, but distinct from each other by their mechanism of action (Lam et al., 2015). siRNAs, in principle, have one target sequence fully complementary to their sequence, and their activity leads to endonucleolytic cleavage of the mRNA. In contrast, miRNAs usually have a mismatched, hairpin structure and target the 3′-untranslated region of the mRNA as well as have multiple targets, as they do not require full complementarity to function. Subsequently, miRNA activity leads to mRNA degradation or suppression of translation, rather than to endonucleolytic activity.

Whether endogenous or exogenous, siRNAs and miRNAs, when larger than 21 nts long (Kim et al., 2005), are cleaved by an endogenous dicer. In humans, the dicer cleaves the siRNAs to 21-23 nt long (Provost et al., 2002). After cleavage, the RNA is loaded to RISC, which mediates the interference or cessation of mRNA translation. siRNAs are mediators of endonucleolytic activity via the argonaut 2 endonuclease, a part of RISC, which requires a high level of complementarity. With double stranded siRNAs, argonaut 2 may use both strands as a guide strand (antisense strand), depending which one has a target in the cell (Rand et al., 2005). After sequence specific cleavage of mRNA, the RISC may continue to degrade the next mRNA complementary to the guide strand sequence.

2.4.2 Small interfering RNAs in antiviral drug development

In essence, RNAi is a regulatory mechanism, but it is also a natural mechanism for antiviral defense, which small interfering RNAs are the natural facilitators of (Ding et al., 2004). Therefore, siRNAs are recognized for their potential in antiviral therapy against hard-to-treat, currently untreatable, or emerging viral infections, as well as against those lacking a vaccine, or in need of novel treatments (Levanova and Poranen, 2018). Moreover, siRNAs are an attractive choice for antiviral therapy, as viral nucleotide sequences are distinct from human sequences. Therefore, finding a viral RNA sequence, whether genomic or gene encoding, to target safely with RNAi

mediating oligonucleotides should be relatively straightforward. This applies not only to the already known viruses, but also to those yet unknown, or not yet clinically relevant. Moreover, the one and only prerequisite for RNAi mediating medicine design is the availability of viral (full genome) sequences, which often either already exist, or may be rapidly generated. For example, the SARS-CoV-2 full genome sequence was available in less than few months after the viral outbreak (Wu et al., 2020).

Various antiviral siRNAs, mostly targeting RNA viruses and none targeting a herpesvirus, are or have been in clinical trials (Levanova and Poranen, 2018). Thus far, however, success stories are lacking. Likely, the reasons lie in escape mutants, RNA stability or delivery, or in a too narrow preclinical understanding of the interaction between RNAi and innate immunity pathways (Levanova and Poranen, 2018). Furthermore, many RNA viruses have been shown to suppress the RNAi pathway (Fabozzi et al., 2011, Li et al., 2016, Qiu et al., 2017). All these aforementioned aspects must be taken into account when designing antiviral siRNA therapy. To begin with, the challenges brought by the complex interactions between RNAi, virus, and the interferon pathways can only be overcome with more profound studies for each compound, preferably both in animals but also *in vitro*, preferably with primary human cells. For solving siRNA stability, chemical modifications are an option. For example, chemical modifications in the 2′-position of the ribose are known to increase siRNA stability against nucleases, which otherwise would degrade the siRNA (Allerson et al., 2005, Manoharan et al., 2011). The chemical modifications also reduce off-target effects (Fedorov et al., 2006, Jackson et al., 2006). For efficient and safe delivery of (si)RNAs many possible approaches, such as different nanoparticles and conjugates exist (Tatiparti et al., 2017), but also siRNAs without delivery reagents have been safe in humans, at least when used as a lung targeted aerosol with intranasal delivery (DeVincenzo et al., 2008). Escape mutants are always a problem in antiviral drug design. In siRNA design, independent of the position, any mismatch decreases silencing activity, whereas a double mutation in the target area will lead to total loss of siRNA activity (Holen et al., 2002, Dahlgren et al., 2008). To overcome any loss-of-function, pooling of various siRNAs is shown a good option (Parsons et al., 2009). Additionally, approaches where numerous active siRNAs are enzymatically cleaved from a thousands of base pairs long dsRNA are being developed (Romanovskaya et al., 2012, Nygårdas et al., 2009). The approach appears to not allow development or emergence of resistance in an *in vitro* setting (Paavilainen et al., 2016).

The challenges related to siRNA drugs should be possible to overcome, especially if fundamental research in the area is supported. Importantly, proving siRNAs as a possible therapy of humans, both functionally and for regulatory authorities, there are already three siRNAs approved by the European Medicines

Agency (EMA) in the market. The first siRNA drug was approved in 2018, and all three are by the same company, Alnylam. These medicines are patisiran (Onpattro®), givosiran (Givlaari®), and lumasiran (Oxlumo®), none of which are antiviral siRNAs, but approved for treatment of polyneuropathy, acute hepatic porphyria, or hyperoxaluria, respectively. They are delivered either intravenously by lipid nanoparticles (patisiran), or subcutaneously (givosiran, lumasiran), enabled by N-acetylgalactosamine (GalNAc)-conjugation ideal for liver delivery. Importantly, all these siRNA medicines contain 2′-F (2′-fluoro) and 2′-OMe nucleotides. Patisiran has 2′-OMe modifications in all pyrimidines of the sense strand, and two in the uridines of the antisense strand, with dTdT overhangs in both strands (EMA/554262/2018). Givosiran and lumasiran both have fully modified antisense and sense strands, with both 2′-OMe and 2′-fluoro modifications represented in different nucleotides in both strands (givosiran: EMA/CHMP/70703/2020, lumasiran: EMA/568312/2020). Such 2′-modifications offer protection from nucleases, do not interfere with RNAi and increase siRNA potency and stability.

For future HSV therapy, RNAi could offer the much needed novel MOA, independent of mutations in TK and in DNA polymerase. In fact, antiviral anti-HSV siRNAs have already shown promise in a handful of *in vivo* studies (Palliser et al., 2006, Li et al., 2014, da Silva et al., 2016, Paavilainen et al., 2017). Importantly, for treatment of HSV-1 infection with siRNA, the delivery routes would not be a major challenge, as the main target tissues would be topical, such as the skin or the eye. Furthermore, for targeting HSE, or even latent or reactivating infection, intranasal delivery of an siRNA could be a viable option (Rodriguez et al., 2018).

2.4.2.1 Beyond small interfering RNAs

In addition to siRNAs, many other classes of molecules, such as miRNAs or antisense DNA oligonucleotides (ASOs), mediate sequence-specific RNA degradation, or prevention of its processing. ASOs are recognized for their potential also in antiviral therapy (Tarn et al., 2021). In fact, an antiviral ASO referred as fomivirsen was approved by the FDA as early as 1998 for treatment of HCMV retinitis in HIV-1 patients, but was withdrawn due to lack of demand as HIV-1 treatment advanced (Perry and Balfour, 1999). Although RNA viruses seem to occupy also the ASO pipeline, recently, an ASO has been highly promising in phase II clinical studies against hepatitis B virus (Yuen et al., 2021), and is now heading towards phase III studies. ASOs appear to not yet be studied for anti-HSV therapy. As for miRNAs, it is widely known that both human and HSV encoded miRNAs have an important role in regulation of the HSV life-cycle, as well as of the host antiviral response. Therefore, miRNA mimics (mimicking intrinsic miRNAs), synthetic miRNAs, or miRNA inhibitors (antagomirs), could have potential in

antiviral RNAi therapy of HSV-1 infection (Lam et al., 2015). In recent studies, both natural and synthetic miRNAs have yielded promising results *in vitro* in inhibition of HSV replication (Wang et al., 2018, Sadegh Ehdaei et al., 2021).

2.4.3 Swarms of small interfering RNA

siRNA swarms are a pool or a collection of double stranded siRNAs, which are enzymatically cleaved from a longer (hundreds to thousands of bps) dsRNA representing either a real, synthetic, or chimeric pathogenic sequence (Jiang et al., 2019, Romanovskaya et al., 2012, Nygårdas et al., 2009). Due to the exceptionally long targeted pathogenic sequence, the siRNA swarm approach is considered to decrease the likelihood of pathogen escape, to ease siRNA target sequence selection, and to diminish off-target effects, as the concentration of each individual siRNA is lower. The enzymatic synthesis of siRNA swarms starting from the pathogen sequence is illustrated in **Figure 3** (please see later in 4.3.1.).

The preferred dicer used for cleavage of the dsRNA in siRNA swarm therapy for HSV-1 is the *Giardia intestinalis* dicer, which cleaves dsRNA into 25-27 bp long dsRNA molecules (Macrae et al., 2006). In contrast to siRNA swarms cleaved with human dicer, the siRNA swarm cleaved with *Giardia intestinalis* dicer had minimal type I and III interferon induction in an *in vitro* study, with or without an HSV-1 infection present (Paavilainen et al., 2015). Furthermore, dicer substrate siRNAs (sometimes referred as DsiRNAs), and especially those 27 nt long, have been shown as most potent siRNAs for RNAi in contrast to shorter RNAs (<21 nt), which are directly loaded to RISC (Kim et al., 2005). After 30 bps, the toxicity of siRNAs begins to elevate (Reynolds et al., 2006), and peaks at 88 bp (Jiang et al., 2011).

Antiviral siRNA swarms have been shown efficient *in vitro* against HSV-1 and HSV-2 (Romanovskaya et al., 2012, Paavilainen et al., 2016, Paavilainen, 2017, Paavilainen et al., 2015), influenza A (Jiang et al., 2019), and enteroviruses (Nygårdas et al., 2009), as well as efficient *in vivo* against HSV-1 (Paavilainen et al., 2017). Against HSV-1, which is the most widely studied virus in siRNA swarm therapy, the best target has been the UL29 essential gene of HSV-1 (Paavilainen et al., 2016). The UL29 targeting siRNA swarm has proven *in vitro* to be well tolerated, to not allow any emergence of resistance over five consecutive treatment passages, to diminish the replication of various clinical isolates with little variation, and to induce only modest type I and type III interferon responses in *in vitro* models, even when both antiviral RNA and HSV-1 are abundant in the cell culture (Paavilainen et al., 2015, Romanovskaya et al., 2012). Furthermore, the UL29 targeted siRNA swarm has demonstrated *in vivo* activity in a corneal model of infection without any delivery reagent with topical application as droplets (Paavilainen et al., 2017).

The siRNA swarms are an advancement to antiviral siRNA therapy, perhaps solving the challenge of pathogen escape. Thus far, especially the UL29 targeted siRNA swarms have shown great promise, and could thus offer the needed candidate for the unmet medical needs of herpes simplex infection. Nevertheless, the siRNA swarms still require further improvement, as an siRNA swarm is still vulnerable to the traditional enemies of any RNA therapy: RNA stability and delivery.

3 Aims

Previously, siRNA swarms were developed to answer to the unmet medical need that is the resistance of HSV-1 to the current antiviral therapies. Here, a novel type of siRNA swarms with incorporated 2′-fluoro-modified adenosines, cytidines, or uridines is introduced for establishing a yet improved candidate for future therapy of HSV-1 infection.

The aim of this doctoral study was to advance the siRNA swarm concept by use of chemically modified siRNA swarms. This study was designed to gain knowledge on their cytotoxicity, on their antiviral efficacy, on the effect they have on host responses, with or without abundant viral challenge, and on how the treatment efficacy compares to that by ACV.

The specific aims of the study were:

1) To validate the modified siRNA swarms in a proof-of-concept cell line, and to select a modified siRNA swarm for further studies.

2) To establish whether ACV sensitivity of the clinical isolates affects siRNA swarm treatment outcome.

3) To determine the antiviral efficacy of and host innate responses to selected modified siRNA swarms, with or without viral challenge, in a self-validated *in vitro* model of infected cornea.

4 Materials and Methods

4.1 Cell lines (I-III)

In this study, three immortalized monolayer cell lines were used. First, green monkey kidney epithelial cells, known as Vero cells (CCL-81, ATCC, Manassas, VA, USA), which naturally lack type I interferon response and are the most common cell line for HSV propagation, were used. They were upkept in M199 (Gibco, Waltham, MA, USA) with 5% fetal bovine serum (FBS) (Serana, Pessin, Germany) and gentamycin (Gibco). Second, a neuroglioma cell line (U373MG), representing cells of the human nervous system, and originally derived from ATCC, was used. The cell line is currently reclassified as U-251 (ECACC 09063001), but referred here as U373MG to allow continuation with previous studies (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016). Third, an immortalized human corneal epithelial (HCE) cell line, which was a generous gift from Arto Urtti, University of Eastern Finland, was used. HCE cells were maintained in DMEM with L-Glucose (4.5 g/L) (Lonza, Basel, Switzerland) supplemented with 7% FBS.

4.2 Viruses (I-III)

In a majority of the experiments, a recombinant HSV-1 strain expressing a green fluorescent protein (GFP) from a mouse cytomegalovirus promoter (HSV1(17+)LoxPmCMVGFP, abbreviated HSV-1-GFP) was used. In addition, reference strains HSV-1 (17+), F, and KOS, as well as another recombinant, fluorescent protein expressing strain (HSV1(17+)Lox-CheVP26, abbreviated HSV-1 mCherry), a thymidine kinase deficient strain (Δ305), and various clinical isolates were used. All used strains and their original references are listed in Table 3. Without exception, the used clinical isolates originated from Finland, from the Department of Virology, University of Turku and had previously been confirmed as HSV-1 with an immunoperoxidase rapid culture assay (Ziegler et al., 1988).

Table 3. Viruses used in this study.

Strain	Type	[1]Genbank #	Original reference
HSV-1 (17+)	Reference strain	JN55585.1	(McGeoch et al., 1986, McGeoch et al., 1988a)
HSV-1 KOS	Reference strain	KT899744.1	(Smith, 1964)
HSV-1 F	Reference strain	GU734771.1	(Ejercito et al., 1968)
HSV-1-GFP	Recombinant, GFP expressing HSV-1	-	(Snijder et al., 2012, Mattila et al., 2015)
HSV-1 mCherry	Recombinant, mCherry expressing HSV-1	-	(Sanbaumhüter et al., 2012)
HSV-1 Δ305	Thymidine kinase deficient HSV-1	-	(Post et al., 1981)
H12114	Finnish clinical HSV-1 isolate	MH999844.1	(Bowen et al., 2019)
H12117	Finnish clinical HSV-1 isolate	MH99845.1	(Bowen et al., 2019)
H12118	Finnish clinical HSV-1 isolate	MH99847.1	(Bowen et al., 2019)
H1215	Finnish clinical HSV-1 isolate	MH99846.1	(Bowen et al., 2019)
H15119	Finnish clinical HSV-1 isolate	MH999850.1	(Bowen et al., 2019)
H12115	Finnish clinical HSV-1 isolate	-	Study II
H12119	Finnish clinical HSV-1 isolate	-	Study II
H15110	Finnish clinical HSV-1 isolate	-	Study II
H15112	Finnish clinical HSV-1 isolate	-	Study II
H15115	Finnish clinical HSV-1 isolate	-	Study II
H15117	Finnish clinical HSV-1 isolate	-	Study II
H1517	Finnish clinical HSV-1 isolate	-	Study II
H1519	Finnish clinical HSV-1 isolate	-	Study II
H151V1	Finnish clinical HSV-1 isolate	-	Study II
H151V2	Finnish clinical HSV-1 isolate	-	Study II
H151V3	Finnish clinical HSV-1 isolate	-	Study II
H151V4	Finnish clinical HSV-1 isolate	-	Study II
V1-V36	Finnish clinical HSV-1 isolates	-	(Kalke et al., 2022)

[1]Genbank accession number is given, when a full genome sequence for the indicated strain is available

A majority of the experiments in this study were conducted with viruses stored in MNT (20nM MES (Sigma), 100mM sodium chloride, 30nM Tris), referred as MNT stocks. MNT stocks were propagated in Vero cells as described before (Romanovskaya et al., 2012). In experiments where clinical isolates beginning with

the letter H were used, all stocks used were supernatant stocks and propagated in Vero cells as described before (Paavilainen et al., 2016). In experiments where clinical isolates V1-V36 were used, viral stocks were such with cells and supernatant collected in milk. The propagation of these stocks, also done in Vero cells, is described before (Kalke et al., 2022).

4.2.1 Plaque titration (I-III)

The viral titers were determined by plaque assay, also referred to as plaque titration. In short, serial dilutions of the samples were added onto Vero cells in DMEM with 2% of heat inactivated (hi)FBS and gentamycin. After a 1-2h incubation, DMEM supplemented with 5-7% hiFBS, gentamycin and 80-160 mg/l of human immunoglobulin G (IgG), KIOVIG (Baxalta, Wien, Austria) was added on top of the pre-existing medium in a 1:1 volume ratio. After 3 to 4 days of incubation, the cell culture supernatant was removed and the cells fixed with +4°C methanol for 5 minutes RT, and stained with 0.1% crystal violet diluted in PBS (GE Healthcare Lifesciences, Marlborough, MA, USA). The crystal violet stained cells were rinsed with tap water and the formed plaques were counted. The presence of human IgG in the cell culture medium allows only lateral spread of the virus, resulting in viral plaques forming in the cell layer. After staining, the plaques are visible even without microscope. The plaques are countable, and allow for use of the quantitative unit plaque forming units (pfu) per volume for concentration of viral samples, or stocks.

4.2.2 Determination of acyclovir sensitivity (II)

ACV sensitivity was studied on Vero cells with a plaque reduction assay, as described before (Bowen et al., 2019). The assay was conducted in a 96-well plate with 100 pfu/well infections. The ACV concentrations ranged from 0.03 to 128 µg/ml, corresponding to approximately 0.1 to 570 µM, respectively. At 2-3 hours post infection (hpi), DMEM supplemented with 5-7% hiFBS, gentamycin and human IgG (80-160 mg/l) was added to the cells. At 3 days post infection (dpi), the cells were fixed and stained as with plaque titration, and subsequently quantified for plaques. For determination of the inhibition of viral growth (%) by ACV, untreated, infected samples were used as a point of comparison. The limit of ACV resistance was considered to be the half maximal inhibitory concentration (IC_{50}) value of 1.9 µg/mL (8.4 µM), as before (Bowen et al., 2019).

4.3 *In vitro* RNAi (I-III)

4.3.1 siRNA swarm synthesis (I, III)

The modified short interfering RNA (siRNA) swarms were synthesized enzymatically. As before (Romanovskaya et al., 2012), the target sequences were first amplified with polymerase chain reaction (PCR) to produce DNA templates for single stranded (ss)RNA production. The amplification primers had recognition sequences for bacteriophage T7 DNA-dependent RNA polymerase (DdRp), or for ϕ6 RNA-dependent RNA polymerase (RdRp) in the 5′-ends. The conversion to ssRNA was done with canonical nucleotides and T7 DdRp, and the subsequent conversion to double stranded (ds)RNA with ϕ6 RdRp and 2′-fluoro-modified nucleotides (#N-1007, 2′-fluoro-2′-dATP; #N-1008, 2′-fluoro-2′-dCTP; #N-1010, 2′-fluoro-2′-dUTP, TriLink Biotechnologies, San Diego, CA, USA). In the ϕ6 RdRp reaction, either 10 or 100% of the adenosines, cytidines, or uridines were modified, and the rest of the nucleotides were canonical, thus creating either fully (100%) or partly (10%) modified complementary dsRNA strands for each indicated nucleotide. The created dsRNA was processed into swarms of 25 base pairs (bp) long blunt-ended dsRNA molecules using the *Giardia intestinalis* Dicer, as before (Romanovskaya et al., 2012), and purified as before (Romanovskaya et al., 2013). For production of unmodified siRNA swarms, the same protocol, without the modified nucleotides, was used. Synthesis of the swarms is visualized in **Figure 3**.

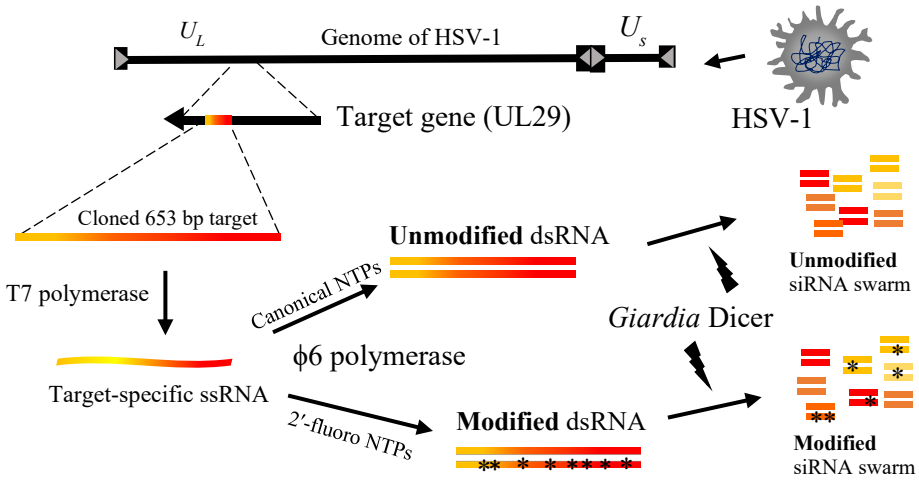


Figure 3. Visualization of the steps for enzymatical synthesis of 2′-fluoro-modified or unmodified siRNA swarms. The example is of synthesis of modified and unmodified UL29-targeted anti-HSV siRNA swarms. The figure is modified from the graphical abstract of study I.

All antiviral siRNA swarms in this study targeted a 653 bp sequence of the essential UL29 gene of HSV-1, as shown most feasible before (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016, Paavilainen et al., 2017). The nonspecific siRNA swarms, used as controls for target specificity of RNA interference (RNAi), targeted either a lac repressor gene or the GFP gene, neither of which are present in wild type HSV, are essential for HSV replication, nor are present in any of the used cell lines. In this thesis, in studies with U373MG cells, the lac repressor gene derived nonspecific siRNA swarm was used, whereas with HCE cells, the nonspecific siRNA swarm derived from the GFP sequence was used. The two nonspecific siRNA swarms were shown antivirally equal and similar in innate response profile in study III, where the GFP targeted siRNA swarms was referred as 'transgene specific'. In addition to the siRNA swarms, a known cytotoxic 88 bp long dsRNA was used as a positive control for cytotoxicity. The synthesis of the 88 bp dsRNA is described in (Jiang et al., 2011). All RNAs are summarized in **Table 4**.

Table 4. RNAs used in this thesis.

Short name of RNA	Type	RNA target sequence	Modification		Reference
			nt	%	
10% F-A	siRNA swarm	UL29 gene of HSV-1, 653 bp sequence	A	10	Study I
100% F-A			A	100	
10% F-C			C	10	
100% F-C			C	100	
10% F-U			U	10	
100% F-U			U	100	
unmodified			None		(Romanovskaya et al., 2012)
100% F-A nonspecific		lac repressor gene of pET32b vector, 401 bp sequence	A	100	This study
nonspecific			None		Study I
		GFP gene of pCR3.1GFP plasmid (Aalto et al., 2007), 717 bp sequence	None		(Romanovskaya et al., 2012)
88 bp dsRNA	dsRNA	bacteriophage ϕ6 S segment, 88 bp sequence	None		(Jiang et al., 2011)

4.3.2 Transfection (I-III)

All cell lines were transfected using Lipofectamine RNAiMAX (#13778075, Thermo Fisher, Waltham, MA, USA) and the manufacturer's protocol for forward transfection. All transfections were performed on 96-well plates (e.g. CLS3595, Sigma Aldrich, St. Louis, MO, USA). The 88 bp dsRNA was always used at 1 pmol/well (10 nM), whereas the other RNAs were used usually with 5 pmol/well (50 nM), depending on the experiment.

4.3.3 Cytotoxicity of RNA (I, III)

The cellular viabilities during siRNA swarm treatment were assessed at 48 hours post transfection (hpt) with a luminescent ATP-based viability assay, CellTiter-Glo (G9681, Promega, Madison, WI, USA) according to the manufacturer's protocol. The luminescence was quantified with VICTOR Nivo Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The relative viability (%) of the cells was determined against untreated cells.

4.3.4 Antiviral RNAi assay settings (I-III)

In this study, many settings for the antiviral RNAi assays were used. The settings represented either a prophylactic or a therapeutic setting, in both of which the infection and treatment were four hours apart by default. In the prophylactic setting, described also before (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016), at 4 hpt, the cells were washed three times with DMEM with 2% hiFBS and gentamycin, infected with viral dilutions made in the same medium, and then at 1-2 hpi washed twice to remove excess virus. In contrast to the prophylactic setting, in the therapeutic setting the cells are treated after the infection. In short, before infection, the cells were washed once with DMEM with 2% hiFBS, and infected with viral dilutions made in the same medium. At 1-2 hpi, the cells were washed twice with serum-free medium (e.g. OptiMEM (Gibco, Waltham, MA, USA)), transfected as per manufacturers protocol (please see 4.3.2.), and washed three times at 4 hpt. In both prophylactic and therapeutic settings, the cells were left in their own culture medium after the final wash. The follow up and sampling were done at the chosen timepoints.
The settings described above were modified in some of the dosing experiments to suit different purposes. In case of such modifications, the changes are described in the corresponding figure legends.

4.3.5 Viral challenge in the antiviral RNAi assays

At the timepoint of infection, the cells were infected on 96-well plates with a clinically relevant viral challenge of 1000 pfu/well, as before (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016). The viral dose corresponds to 10 000 pfu/ml, as the infection volume was 100 µl, or to approximately 0.06 and 0.14 pfu/cell (= multiplicity of infection (MOI)) in U373MG and HCE cells, respectively. With the antiviral RNAi assays in Vero cells, the infection was 100 pfu/well, which corresponds to 1000 pfu/ml, or to approximately 0.007 pfu/cell.

4.3.6 Sample collection and preservation (I-III)

Supernatant samples were collected to a separate, parallel, preferably U-bottom 96-well plates (e.g. CLS3799, Sigma Aldrich), and placed to -80°C to await titration. After removing the supernatant, the cells were covered with 80-100µl of TRIzol reagent (#15596026, Thermo Fisher) in the original plate, and placed to -80°C to await total cellular RNA extraction, if feasible. Thawing of the plates was done in RT or in +4°C.

4.4 Reverse Transcriptase quantitative PCR (RT-qPCR) (I-III)

The total cellular RNA was extracted, processed to complementary (c)DNA, and the cDNA amounts were subsequently quantified, as before (Romanovskaya et al., 2012, Paavilainen et al., 2015). In short, the total cellular RNA was extracted from samples covered with TRIzol according to the manufacturer's protocol. The RNA was then treated with DNase (EN0521, Thermo Fisher) and processed into cDNA with RevertAid H Reverse Transcriptase (EP0441, Thermo Fisher) and random hexamer primers (SO142, Thermo Fisher). The target gene specific cDNA amounts were quantified using detection primers (**Table 5**) and SYBR Green enzyme (K0253, Thermo Fisher). The quantitative PCR was run with QIAGEN Rotor-Gene Q (2-Plex) and Rotor-Gene Q Software 2.3.1.49. Each data point was normalized to housekeeping gene (GAPDH) expression of the same sample. The quantity standards, or calibrators, specific for each primer pair, were used in RT-qPCR analysis for quantification. The quantity standards, used with tenfold dilutions of 10^8 to 10^0 copies per qPCR reaction were either plasmids including the target gene (**Table 5**), or DNA sequences including the target, which were amplified from cDNA of human cells with specific primers. The used primer pairs for detection as well as the plasmids used as quantity standards or the primer pairs used to generate the quantity standards, are summarized for each target gene in **Table 5**.

Table 5. Quantity standards and detection primers for qPCR. For each RT-qPCR target, the sense and antisense sequences for each detection primer pair, and the quantity standard template or primers used to create the quantity standard template, are listed. All primer sequences are listed as 5′ -3′.

Target	Quantity standards and detection primers for qPCR			Reference
GAPDH	Quantity standard	forward	AAT CCC ATC ACC ATC TTC CA	(Nygårdas et al., 2009)
		reverse	TGA GTC CTT CCA CGA TAC CA	
	Detection primers	sense	GAG AAG GCT GGG GCT CAT	
		antisense	TGC TGA TGA TCT TGA GGC TG	
IFN-β	Quantity standard	forward	AGA CTG CTC ATG CGT TTT CC	(Peri et al., 2008)
		reverse	TCC TCC AAA TTG CTC TCC TG	
	Detection primers	sense	TCT CCA CGA CAG CTC TTT CCA	
		antisense	ACA CTG ACA ATT GCT GCT TCT TTG	
ISG54	Quantity standard	forward	AAG CCA CAA TGT GCA ACC AT	(Romanovskaya et al., 2012)
		reverse	GAG CCT TCT CAA AGC ACA CC	
	Detection primers	sense	ACT ATC ACA TGG GCC GAC TC	
		antisense	TTT AAC CGT GTC CAC CCT TC	
MxB	Quantity standard	forward	CTG AAC GTG CAG CGA GCT T	Study III
		reverse	TCG ATG AGG TCA ATG CAG GG	
	Detection primers	sense	GGA AAG CAG CGT CCT TCT CT	
		antisense	ATT CCT TCC AGC AAC AGC CA	
TLR3	Quantity standard	forward	ATG AAA TGT CTG GAT TTG GAC TA	(Peri et al., 2008)
		reverse	GTT AGC TGG CTA TAC CTT GTG A	
	Detection primers	sense	TAG CAG TCA TCC AAC AGA ATC AT	
		antisense	AAT CTT CTG AGT TGA TTA TGG GTA A	
U_S1	Quantity standard	forward	AAG CCC AAA TGC AAT GCT AC	(Paavilainen et al., 2016)
		reverse	CAG ACA CTT GCG GTC TTC TG	
	Detection primers	sense	CAT GCG CCA GTG TAT CAA TC	
		antisense	CGG CAG TAT CCC ATC AGG TA	
U_L29	Quantity standard	forward	GGT GCG GTC AAA AAT AAG GA	(Romanovskaya et al., 2012)
		reverse	CCT ACC AGA AGC CCG ACA AG	
	Detection primers	sense	AAG CTG GTT GCG TTG GAG	
		antisense	TTT CTG CTG AAG CAG TTC CA	
U_L48	Quantity standard	the plasmid clone pRB3717 (McKnight et al., 1987)		(Broberg et al., 2003)
	Detection primers	sense	TTT GAC CCG CGA GAT CCT AT	
		antisense	GCT CCG TTG ACG AAC ATG AA	
IL-29	Quantity standard	pUNO1-hIL-29 (InvivoGen)		(Paavilainen et al., 2016)
	Detection primers	sense	GAC GCC TTG GAA GAG TCA C	
		antisense	CTC ACC TGG AGA AGC CTC A	
MxA	Quantity standard	a cDNA clone of human MxA (Yahya et al., 2017)		(Yahya et al., 2017); III
	Detection primers	sense	GAG GAG ATC TTT CAG CAC CTG	
		antisense	TGG ATG ATC AAA GGG ATG TG	
IFN-κ	Quantity standard	pUNO1-hIFNK (InvivoGen)		Study III
	Detection primers	sense	CAG AAA CTC TTG GGG CAA CTC	
		antisense	TCA CCT GAG AAG AGT CAC CTG	

5 Results

5.1 Cytotoxicity of modified siRNA swarms (I, III)

The modified UL29-targeted siRNA swarms harbored either 2′-fluoro-modified adenosines (A), cytidines (C), or uridines (U), fully (100%) or partly (10%) in their sequence. Accordingly, the modified UL29-targeted siRNA swarms are referred to as 10 or 100% F-N, where N is the modified nucleotide, and the 10 or 100% refer to the sequence being either partly or fully modified, respectively, for the indicated nucleotide. (For a summary of the RNAs used in this thesis, please see Table 4.) The possible cytotoxicity of any of the modified siRNA swarms was checked in U373MG and HCE cells at a 50 nM concentration (**Figure 4A**). The unmodified UL29-targeted siRNA swarm, nonspecific siRNA swarm, transfection with transfection reagent only (mock treatment), and the known cytotoxic 88 bp dsRNA were included as controls. The cytotoxic 88 bp dsRNA decreased the relative cellular viability significantly in contrast to mock treatment in both cell lines (**Figure 4A**). In U373MG cells, no siRNA swarm was significantly more cytotoxic than mock treatment, nor was the relative viability after treatment with any the siRNA swarms below 80% (**Figure 4A**), which is the limit considered as an acceptable relative viability after treatment (Romanovskaya et al., 2012). In HCE cells, 100% F-C, 10% F-C, and 10% F-U were significantly more cytotoxic than mock treatment, and treatment with the 100% F-C decreased the relative viability of HCE cells well below the 80% limit (**Figure 4A**).

The cytotoxicity of the fully and partly 2′-fluoro-modified UL29-targeted siRNA swarms was studied further in a concentration range of 0 to 150 nM (**Figure 4B**). The unmodified siRNA swarm was used as a control. In HCE cells, the relative viability was similar throughout the dose range, whereas with U373MG cells, the relative viability dropped between the 100 nM and 150 nM doses (**Figure 4B**). In both cell lines, the curves of relative viability were relatively equal for fully modified, partly modified, and for unmodified UL29-targeted siRNA swarms throughout the dose range (**Figure 4B**).

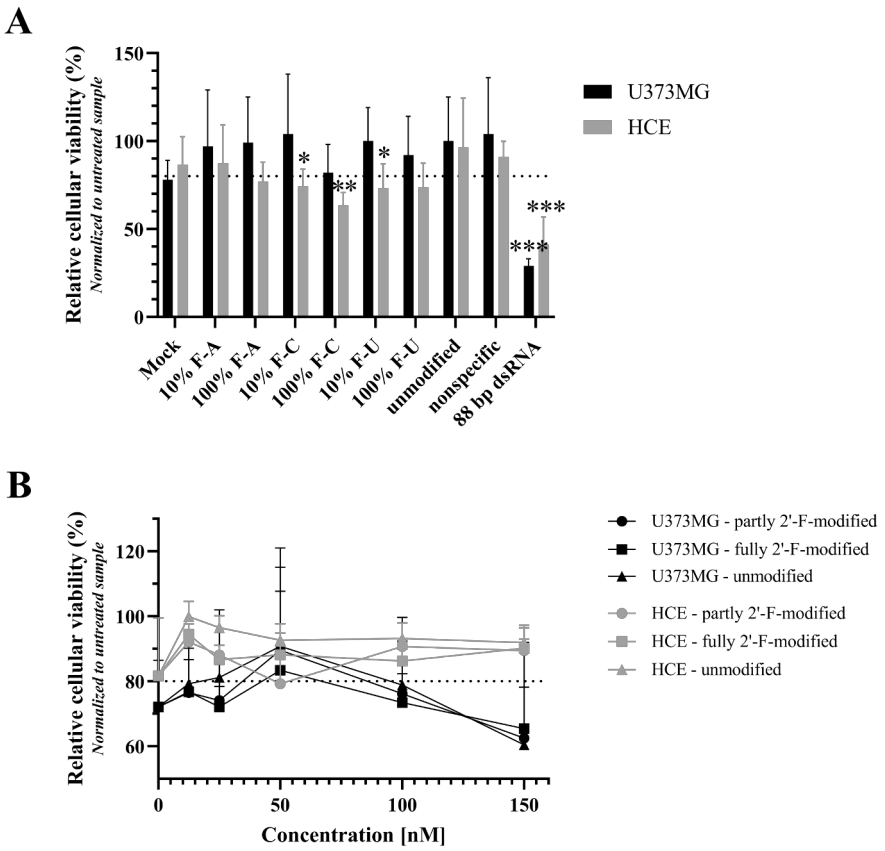


Figure 4. Viability of U373MG and HCE cells after treatment with modified siRNA swarms. U373MG and HCE cells were transfected with the indicated siRNA swarms. The modified siRNA swarms (10% F-N or 100% F-N) harbored 2′-fluoro-modified adenosines (A), cytidines (C), or uridines (U), fully (100%) or partly (10%) in their sequences. In addition to the modified siRNA swarms, unmodified HSV-targeted and nonspecific siRNA swarms, as well as a known positive control for cytotoxicity, 88 bp dsRNA, were included as controls. The relative viabilities were determined at 48 hours post transfection (hpt) and are shown against mock treatment, referring to transfection with transfection reagent alone. The dashed line represents the relative viability of 80%, which has been previously considered as the limit of acceptable cytotoxicity (Romanovskaya et al., 2012). **(A)** The relative cellular viabilities were determined after treatment with each of the indicated siRNA swarms at a 50 nM concentration. The concentration for the 88 bp dsRNA was 10 nM. The columns represent the mean and the whiskers the standard deviation. The data is from two repeated experiments with four parallels each. The statistical significances were determined by pairwise comparisons (Mann Whitney U test) against the mock treated cells. The p-values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) are shown when the viability was significantly lower than mock treatment. **(B)** The relative cellular viabilities were determined after transfection with 12.5, 25, 50, 100, or 150 nM of unmodified, or fully (10%) or partly (100%) 2′-fluoro-uridine modified HSV-targeted siRNA swarms (10% F-U, 100% F-U, respectively). The data points represent the mean and the whiskers the standard deviation. The data is from four or more biological replicates.

5.2 Antiviral efficacy of the modified siRNA swarms (I, III)

The prophylactic antiviral efficacy of the modified siRNA swarms was studied by live-cell fluorescent imaging enabled by the GFP transgene of the virus (**Figure 5A**) as well as by quantifying viral shedding by plaque assay (**Figure 5B**) and by quantifying viral transcripts via RT-qPCR (**Figure 6**). The endpoint of all aforementioned assays was at 48 hpt (44 hpi) and the siRNA swarms were used at the concentration of 50 nM. In both U373MG and HCE cell lines, treatment with modified (100% F-A or 10% F-A) and unmodified antiviral UL29-targeted siRNA swarms resulted in low or sparse GFP signal from the virus infected wells, whereas after mock treatment, the GFP signal was abundant (**Figure 5A**). This result was confirmed by the significant decrease in viral shedding in all UL29 siRNA swarm treated cells (unmodified, 10% F-A, and 100% F-A) in contrast to mock treated cells (**Figure 5B, C**). The UL29-targeted antiviral siRNA swarms did not differ from each other with statistical significance (**Figure 5B, C**). However, in both cell types, 100% F-A was most significantly antiviral in contrast to mock treatment, followed by 10% F-A, followed by the unmodified siRNA swarm (**Figure 5B, C**).

The viral transcript levels for US1, UL29, and UL48 were quantified in U373MG cells and in HCE cells by RT-qPCR after treatment with UL29-targeted modified (10% F-A/C/U, 100% F-A/C/U), UL29-targeted unmodified, or nonspecific siRNA swarm (**Figure 6**). In U373MG cells, all of the modified siRNA swarms were used, but in HCE cells, only the adenosine modified siRNA swarms were used. In both cell lines, all UL29-targeted siRNA swarms significantly reduced the amount of all three studied viral transcripts in contrast to mock treatment, whereas the nonspecific siRNA swarm did not (**Figure 6**). Moreover, in neither cell line nor with any of the studied viral transcripts were the UL29-targeted siRNA swarms different from each other (**Figure 6**). However, in both cell lines, all used modified siRNA swarms had equal or higher statistical significances against mock treatment, than the unmodified siRNA swarm (**Figure 6G**).

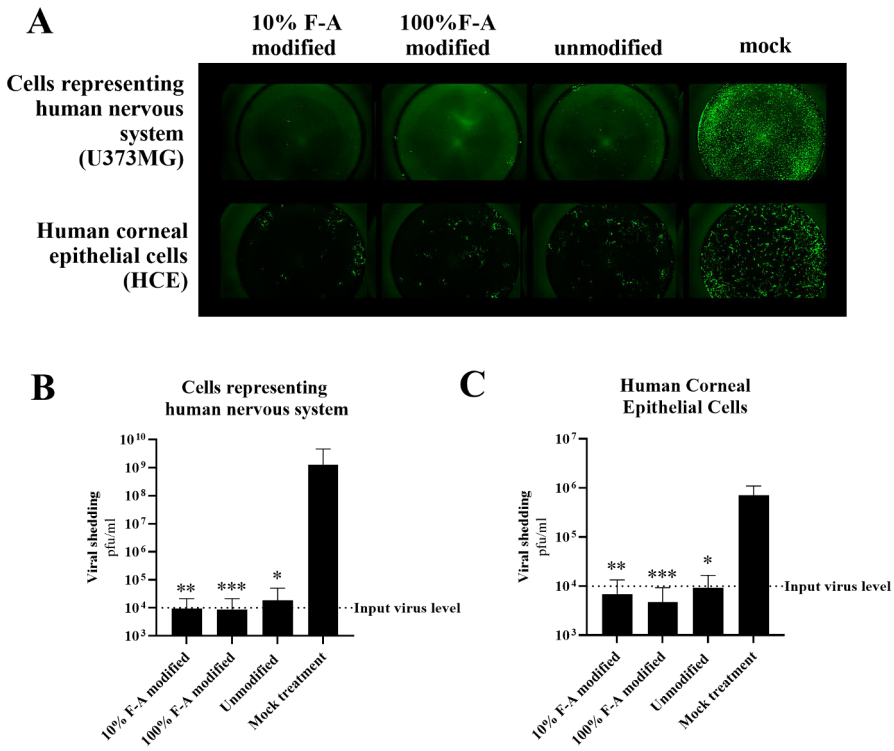
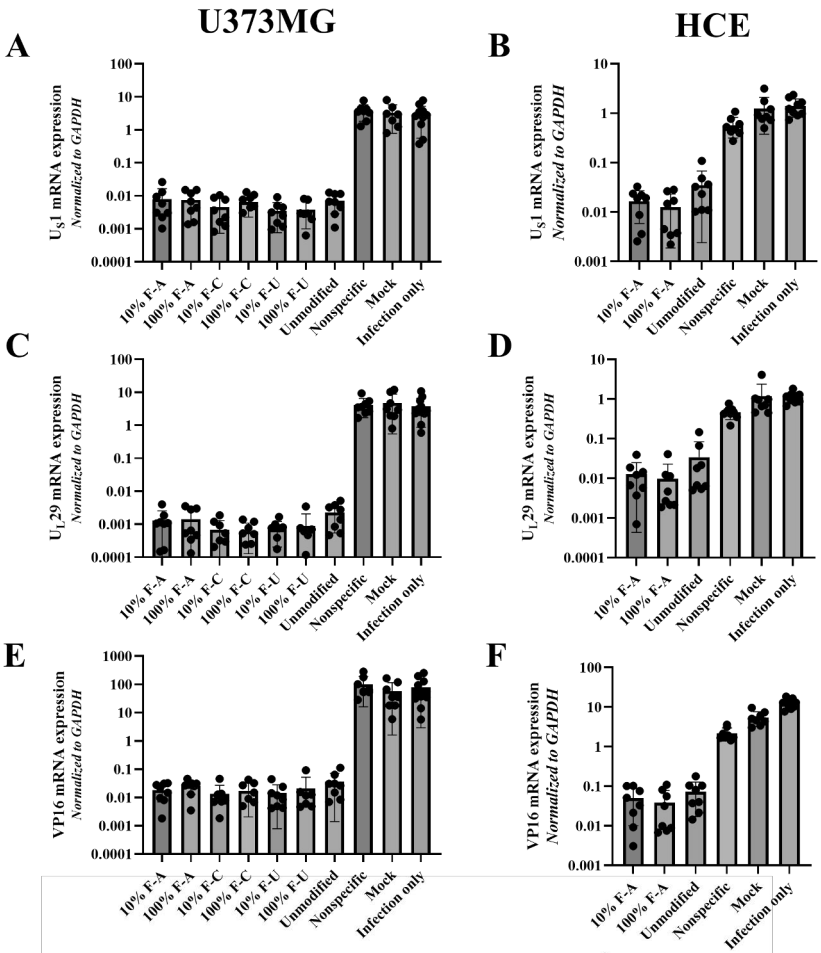


Figure 5. Antiviral efficacy of 10% F-A and 100% F-A siRNA swarms in U373MG and in HCE cells. The cells were treated with 50 nM of 10% F-A, 100% F-A, or unmodified antiviral siRNA swarms, or mock treated with transfection reagent alone. At 4 hours post treatment (hpt) the cells were infected with 1000 pfu/well on 96-well plates of HSV-1-GFP. **(A)** At 48 hpt, the cells were imaged for green fluorescent protein (GFP) signal. The microscope settings were kept the same when scanning trough the wells. All images within a cell line have been adjusted equally. **(B, C)** At 48 hpt, the supernatants of the treated and infected U373MG (B) cells and of the treated and infected HCE cells (C) were collected and plaque titrated to quantify for viral shedding. The dashed line in the figures marks the amount of input virus (1000 pfu/well). The columns represent the mean titer of the treatment group, and the whiskers the standard deviation. The data represents two individual experiments with four parallels each. The statistical significances were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons. All significant statistical differences found were against the mock treated group, and are marked here with asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).



G

		10% F-A	100% F-A	10% F-C	100% F-C	10% F-U	100% F-U	Unmodified	Nonspecific	Infection only	Mock (point of comparison)
U373MG	US1	**	**	***	**	***	***	**	ns	ns	-
	UL29	**	**	***	***	***	***	*	ns	ns	-
	VP16	**	*	***	***	***	***	*	ns	ns	-
HCE	US1	***	***	-	-	-	-	*	ns	ns	-
	UL29	**	***	-	-	-	-	**	ns	ns	-
	VP16	**	**	-	-	-	-	*	ns	ns	-

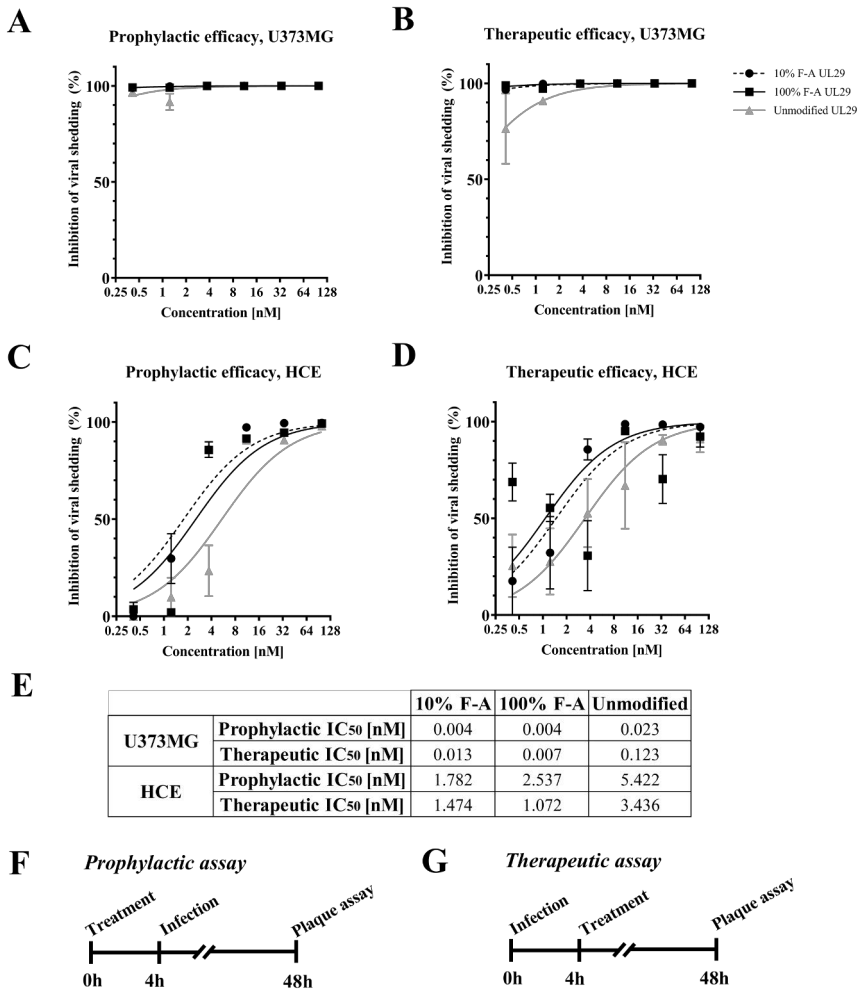
Figure 6. Viral mRNA expression in HCE and U373MG cells after treatment with modified siRNA swarms. U373MG and HCE cells were treated with the modified or unmodified antiviral siRNA swarms, nonspecific siRNA swarm, mock treated, or left untreated. With U373MG cells, all modified siRNA swarms (10% F-A/C/U, 100% F-A/C/U) were used, whereas with HCE cells, only 10% F-A and 100% F-A were used. At 4 hpt, the cells were infected with 1000 pfu of HSV-1-GFP per well on 96-well plates, and at 48 hpt they were quantified for viral transcript, namely US1, UL29, or VP16, expression: **(A)** US1 in U373MG cells, **(B)** US1 in HCE cells, **(C)** UL29 in U373MG cells, **(D)** UL29 in HCE cells, **(E)** VP16 in U373MG cells, and **(F)** VP16 in HCE cells. The columns in each panel represent the mean titer of the treatment group, and the whiskers the standard deviation.

The data points are shown as black spheres. The data is from two individual experiments with four parallels each. **(G)** For clarity, the statistical significances are shown in the table in panel G, and not on the columns in panels A-F. The statistical significances were determined by Kruskal-Wallis test and Dunn's multiple comparisons. The antiviral siRNA swarms were not significantly different to each other in any of the panels. Hence, only the statistical differences against mock treatment are shown, and marked in the table with asterisks (ns, nonsignificant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

The dose-responsiveness of inhibition of viral shedding to increasing concentrations (0.4 to 100 nM) of 10% F-A, 100% F-A, or unmodified UL29-targeted siRNA swarms was determined in both U373MG and HCE cells (**Figure 7**). The dose-responsiveness was determined in both prophylactic and therapeutic assays: in the prophylactic assay, the cells were treated with 50 nM of the indicated siRNA swarm, and infected 4 hours later. In the therapeutic assay, the cells were first infected, followed by treatment 4 hours later. The timeframes for the prophylactic and therapeutic assays are illustrated in **Figure 7G** and **7F**, respectively.

In U373MG cells, with both prophylactic and therapeutic settings, the inhibition of viral shedding by both modified siRNA swarms was more than 98% throughout the dose range (**Figure 7A, B**). At higher doses (>2 nM), the unmodified siRNA swarm was antiviral at the same level as the modified siRNA swarms, but at the lowest doses (<2 nM), its efficacy was not as high (**Figure 7A, B**). The lower potency of the unmodified siRNA swarm was more evident in the therapeutic assay, where the IC_{50} value of the unmodified siRNA swarm was more than tenfold higher than that of the modified siRNA swarms, whereas in the prophylactic assay the same difference was roughly six-fold (**Figure 7E**). However, no significant statistical differences between the antiviral siRNA swarms was found.

In HCE cells, the inhibition of viral shedding by all used antiviral siRNA swarms decreased to less than 30% in the used dose range, and the inhibition levels were close to 100% only at the highest concentrations (**Figure 7C, D**). The potency of the unmodified siRNA swarm was lower than that of the modified siRNA swarms in both prophylactic and therapeutic assays (**Figure 7E**), but again, no significant differences between the antiviral siRNA swarms were found. As in U373MG cells, in HCE cells, the IC_{50} values of the 10% and 100% F-A siRNA swarms were also similar (**Figure 7E**).



		10% F-A	100% F-A	Unmodified
U373MG	Prophylactic IC_{50} [nM]	0.004	0.004	0.023
	Therapeutic IC_{50} [nM]	0.013	0.007	0.123
HCE	Prophylactic IC_{50} [nM]	1.782	2.537	5.422
	Therapeutic IC_{50} [nM]	1.474	1.072	3.436

Figure 7. Dose-response of antiviral siRNA swarm concentration (nM) and HSV-1 infection inhibition (%). **(A)** Prophylactic dose-response in U373MG cells. **(B)** Therapeutic dose-response in U373MG cells. **(C)** Prophylactic dose-response in HCE cells. **(D)** Therapeutic dose-response in HCE cells. **(E)** The IC_{50} values from panels A-D. U373MG cells and HCE cells were treated with 0.41–100 nM (three-fold dilution series) of either 10% F-A, 100% F-A, or unmodified siRNA swarm. In the prophylactic assays (as illustrated in **(F)**), the treated cells were infected with 1000 pfu of HSV-1-GFP at 4 hours post treatment (hpt). In the therapeutic assays (as illustrated in **(G)**), the cells were infected with 1000 pfu of HSV-1-GFP 4 hours before treatment. In both assays, at 48 hpt, the supernatants were collected and determined for viral shedding by plaque assay. The inhibition of viral shedding (%) was determined against mock treated cells. The data was fit using a nonlinear fit. The x-axis is in log_2-scale. Each data point represents the mean of 4 parallels, and the whiskers the standard error of mean of the parallels. The statistical significances within each concentration were checked with Kruskal Wallis test, which was nonsignificant.

The duration of antiviral efficacy of the modified (10% F-C, 100% F-C) and unmodified siRNA swarms was studied in U373MG and in HCE cells (**Figure 8**). In the assay, the cells were treated with 50 nM of the siRNA swarms, and infected at 4, 24, 48, 72, or 96 hpt. In U373MG cells, the inhibition of viral shedding was constantly over 95% and did not show any decrease in response to the lengthened time between the treatment and infection (**Figure 8A**). In HCE cells, the inhibition of viral shedding was likewise constant at more than 95%, when the infection took place at 72 hpt, or earlier (**Figure 8B**). However, independent of the antiviral siRNA swarm, when the cells were infected at 96 hpt, the inhibition of viral shedding decreased evidently in contrast to infection at earlier time points (**Figure 8B**). In either of the cell lines, no statistical differences between the different antiviral siRNA swarms were detected (**Figure 8**).

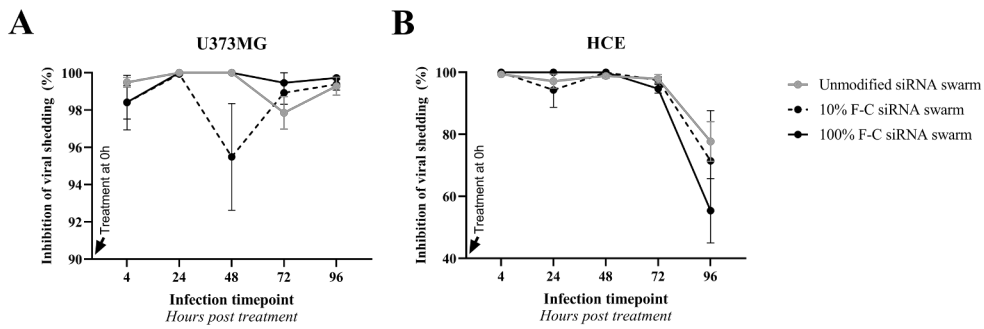


Figure 8. Duration of efficacy of siRNA swarms in HCE and U373MG cells. **(A)** U373MG and **(B)** HCE cells were treated with 50 nM of 10% F-C, 100% F-C or unmodified siRNA swarms, and subsequently infected at 4, 24, 48, 72, or 96 hours post treatment with 1000 pfu of HSV-1-GFP per well on 96-well plates. At 48 hours post infection, the supernatant was collected and quantified for viral shedding using the plaque assay. The inhibition of viral shedding was determined against the viral shedding from cells treated with nonspecific siRNA swarm. The data points represent the mean of four biological replicates and the whiskers their standard deviation. Any statistical differences within each timepoint of infection were checked with Kruskal Wallis test, which was nonsignificant.

The modified (10% F-C, 100% F-C) and unmodified UL29-targeted siRNA swarms were used in U373MG and HCE cells to study whether a repeated viral challenge would affect treatment outcome, and whether a repeated treatment would be beneficial (**Figure 9**). The repeated viral challenge and the repeated treatment were both done at 2 days after the first infection, and the data is shown from 4 days after the first infection. Both scenarios were studied in the therapeutic setting, meaning that the transfection was done 4 hours after the (first) infection. In the not altered, canonical therapeutic setting (grey bars, **Figure 9**), from which the data is also shown from 4 dpi, the antiviral siRNA swarms were similar to each other,

reaching close to 100% inhibition of viral shedding in both cell lines. In U373MG cells, with repeated viral challenge (black bars, **Figure 9A**), the inhibition of viral shedding remained at a similar level to the canonical setting. However, in HCE cells, after repeated viral challenge, the inhibition of viral shedding by the unmodified siRNA swarm was significantly less than that by the 100% F-C (black bars, **Figure 9B**). The repeated treatment did not significantly affect the inhibition of viral shedding in either cell line (white bars, **Figure 9**). For clarity, the assay timeframes are illustrated in **Figure 9C**.

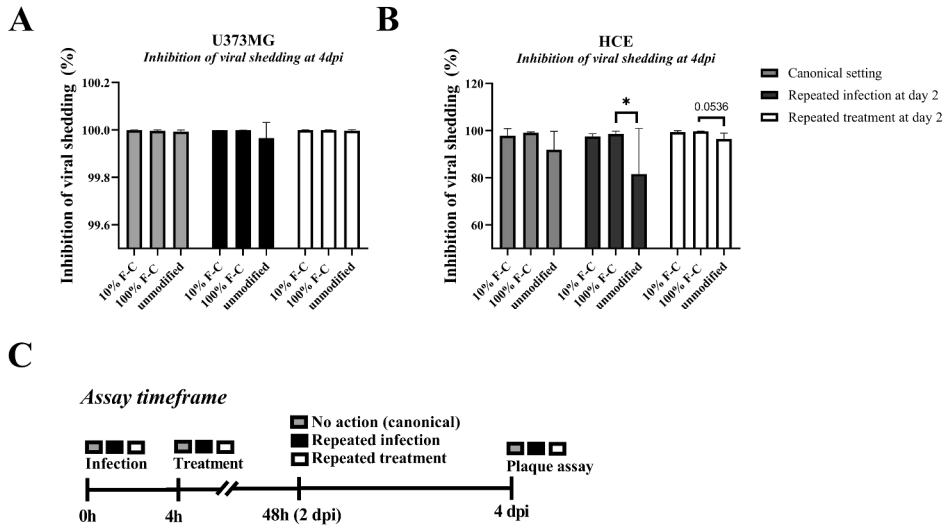


Figure 9. The inhibition of viral shedding by siRNA swarms after repeated viral challenge or repeated dosing. **(A)** U373MG and **(B)** HCE cells were infected with 1000 pfu per well of HSV-1-GFP on 96-well plates. The cells were subsequently treated with 50 nM of 10% F-C, 100% F-C, or unmodified siRNA swarm at 4 hours post infection. At 2 days post infection, the cells were either re-infected with HSV-1 mCherry (black bars), retreated with 50 nM of the same siRNA swarm (white bars), or left as they are (grey bars). The assay timeframes are illustrated in **(C)**. At 4 days post the (first) infection, the supernatants were collected and quantified for viral shedding with a plaque assay. The inhibition of viral shedding (%) is determined against treatment with the nonspecific siRNA swarm. Any statistical significances were checked with Kruskal Wallis test and Dunn's pairwise comparisons within the settings, as well as across the settings for each individual treatment. All statistical significances found are shown (*, $p<0.05$).

5.3 Sensitivity of clinical isolates to siRNA swarms and to acyclovir (II)

The sensitivities (IC_{50}-values) of seventeen clinical HSV-1 isolates, three reference strains (F, KOS and 17+), as well as of an ACV-resistant thymidine kinase deficient strain (Δ305) were determined for ACV and for the unmodified siRNA swarm in

Vero cells (**Figure 10**). The readouts for both antivirals were at 72 hpt, and the concentration ranged from 0.1 to 570 µM for ACV, and from 0.4 to 100 nM for the unmodified siRNA swarm. All strains, except Δ305, were sensitive to ACV, with the sensitivities ranging from 349.7 to 1911.2 nM (corresponding to 0.08 to 0.43 µg/ml). The UL29 siRNA swarm sensitivities ranged from 0.5 nM to 3.0 nM, with no strain being a clear outlier (**Figure 10**). The Δ305 had an ACV IC_{50}-value of 25,500 nM, clearly exceeding the limit of ACV resistance (8400 nM, corresponding to 1.9 µg/ml), but was however susceptible to treatment with UL29 siRNA swarm with an IC_{50} of 1 nM (**Figure 10**).

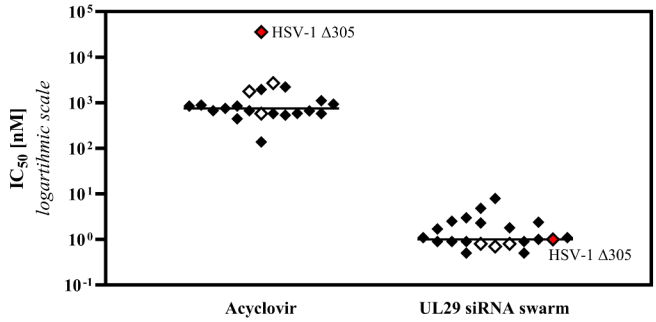


Figure 10. Acyclovir (ACV) and siRNA swarm sensitivity of Finnish clinical HSV-1 isolates. Seventeen clinical isolates[#] (black data points), three reference strains (KOS, F, 17+; white data points) and a thymidine kinase deficient ACV-resistant control HSV-1 Δ305 (red data point) were analyzed for their sensitivity to ACV and to the unmodified antiviral UL29-targeted siRNA swarm. Both sensitivities were determined in Vero cells with 100 pfu per well in 96-well plates with the endpoint at 72 hours post treatment (hpt). For the siRNA swarm, the concentration ranged from 0.4 to 100 nM, and for ACV from 0.1 to 570 µM (100 to 570,000 nM). The IC50 values (nM) were determined based on dose-response analysis between antiviral concentration and the inhibition percentages (%), which were calculated in comparison to untreated cells for ACV and to mock-treated cells for the UL29 siRNA swarm. In the ACV sensitivity assay, the viral strains were analyzed in duplicates for each concentration, whereas in the UL29 siRNA swarm assay, there were five or more biological replicates for each concentration. [#]The clinical isolates used in this analysis are the ones beginning with the letter H in Table 3 in the Methods section.

The susceptibility of thirty-six clinical isolates, 17+, KOS, and F, to the 100% F-A UL29-targeted modified siRNA swarm were determined in U373MG cells (**Figure 11**). In the assay, the canonical, prophylactic setting at a 50 nM concentration was used. The inhibition of viral shedding was determined against a nonspecific control, which in this assay, had 100% of 2′-fluoro-modified adenosines (nonspecific 100% F-A). Altogether, the UL29-targeted 100% F-A was significantly more antiviral than the nonspecific 100% F-A siRNA swarm, with a more than 10,000-fold lower mean in viral shedding (**Figure 11A**). The UL29-targeted 100%

F-A modified siRNA swarm inhibited 100.00% of viral shedding of almost all of the studied strains, with only few of the clinical isolates, V7, V12, V24, and V27, producing any plaques (**Figure 11B**). However, even for these strains, the inhibition of viral release was at least 99.78% (**Figure 11B**).

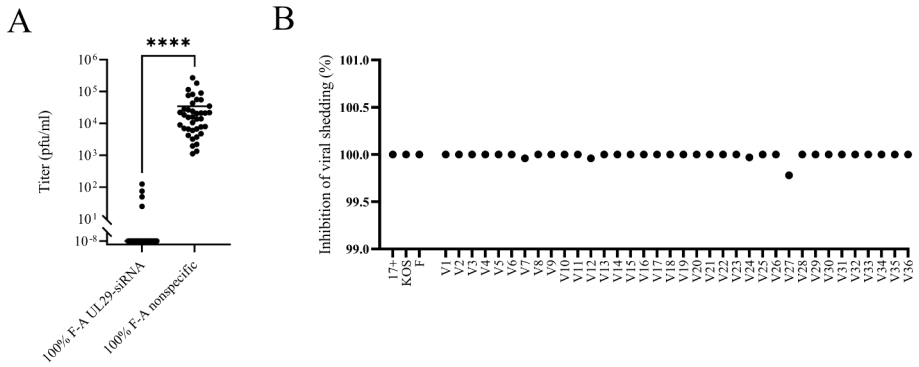


Figure 11. Susceptibility of Finnish clinical isolates to a modified antiviral UL29-siRNA swarm. U373MG cells were treated on a 96-well plate with a modified HSV-specific UL29-siRNA swarm (100% F-A) or a modified nonspecific siRNA swarm (nonspecific 100% F-A), both fully harboring 2′-fluoro-modified adenosines. The cells were subsequently infected with 1000 pfu of each strain (V1-V36, 17+, KOS or F), and the titer of each virus was measured with a plaque assay from the culture supernatant at two days post infection. **(A)** All isolates are shown grouped based on treatment. Each data point represents one viral strain (N≥4 per treatment for each isolate) and the line the mean of the titer of the treatment group. The statistical significance was determined with Mann Whitney pairwise comparison (****, $p<0.0001$). In order to show each data point on the logarithmic scale, the data points with the value 0 were transformed to 1 x 10^{-8}. **(B)** The inhibition of viral shedding for each individual strain. The inhibition of viral shedding (%) is derived from comparing the viral shedding resulting from treatment with the HSV-specific UL29-siRNA swarm to that resulting from treatment with the nonspecific siRNA swarm (N≥4 per treatment for each isolate).

5.4 Host innate responses to treatment with modified siRNA swarms (I, III)

The innate responses of U373MG (**Table 6**) and HCE cells (**Table 7**) to modified antiviral siRNA swarms were determined with and without viral challenge. The timepoints for analysis were 8, 24, and 48 hpt, which correspond to 4, 20, and 44 hpi in the setting where the cells were infected. In both cell lines, the expression of interferon beta (IFN-β), interferon lambda 1 (IFN-λ1; IL-29), interferon stimulated gene 54 (ISG54), human myxovirus resistance protein A (MxA), human myxovirus resistance protein B (MxB), toll-like receptor 3 (TLR3) and interferon kappa (IFN-κ) mRNA expression levels were quantified by RT-qPCR. Before analysis, all data was normalized to housekeeping gene (GAPDH) expression. The innate response data for U373MG cells (**Table 6**) and for HCE cells (**Table 7**) is presented as fold

change to untreated cells, which were either not infected (**Table 6A**, **Table 7A**) or infected (**Table 6B, Table 7B**). The statistical differences of each treatment group in each timepoint were determined against the respective data from the unmodified UL29-targeted siRNA swarm. The presented fold-change values (mean ± SD) are colored based on their (statistical) difference to unmodified UL29-targeted siRNA swarm, as indicated in the table legend. The relative sum of the statistical differences to unmodified UL29 siRNA swarm (SUM (p) in Tables 6 and 7) were determined for each treatment group: if the expression of an innate marker in a timepoint was significantly higher than that induced by the unmodified siRNA swarm, the value added was 1, 2 or, 3, depending whether the p-value was <0.05, <0.01, or <0.001, respectively. If the expression of an innate marker in a timepoint was significantly less than that by the unmodified siRNA swarm, the value added was -1, -2, or -3, depending on whether the p-value was <0.05, <0.01, or <0.001, respectively.

In U373MG cells all modified siRNA swarms (10% F-A, 100% F-A, 10% F-C, 100% F-C, 10% F-U, and 100% F-U), as well as the unmodified siRNA swarm, the nonspecific siRNA swarm, and mock treatment were studied for their innate immunity profile in treated and in treated, infected cells (**Table 6**). Altogether, the innate induction profiles of the treatments were similar in uninfected (**Table 6A**) and in infected (**Table 6B**) cells, as the color profiles were similar between the Tables 6A and 6B. In both settings, especially cytidine and uridine modified siRNA swarms led to significant elevations of innate responses, whereas the 100% F-A, nonspecific, and mock treatment groups were less immunostimulatory in contrast to the unmodified siRNA swarm (**Table 6**). This was reflected also by the relative sums of significances (SUM (p)), which was negative only for 100% F-A, nonspecific, and mock treatment groups. The 10% modified siRNA swarms were either slightly positive or close to 0 by their relative sum of significances, whereas the 100% F-C and 100% F-U had the highest values.

In HCE cells, 10% F-A, 100% F-A, and unmodified UL29-targeted siRNA swarms, as well as the nonspecific siRNA swarm, were studied for their innate immunity profile in treated and in treated, infected cells (**Table 7**). Altogether, in the uninfected cells, 10% F-A was significantly more immunostimulatory than the unmodified siRNA swarm in all innate responses but IFN-κ, whereas the 100% F-A and nonspecific siRNA swarms were similar to the unmodified siRNA swarm (**Table 7A**). In the infected cells, the results were in line to those with uninfected cells, but 10% F-A was not as drastically more immunostimulatory than the unmodified siRNA swarm, as there were statistical differences only in IL-29 and ISG54 expression profiles (**Table 7B**). The relative sums of significances (SUM (p)), reflected the situation, as the 100% F-A and nonspecific siRNA swarms were either at 0 (uninfected) or -1 (infected), whereas mock treatment was highly negative (-23 or -36), and the 10% F-A was the highest positive at 22 (uninfected) or 7 (infected).

Table 6. Innate responses of U373MG cells to **(A)** modified siRNA swarms and to **(B)** modified siRNA swarms and viral challenge. Treatments were done with 50 nM of the indicated RNA, and the infection with HSV-1-GFP at 4 hpt with 1000 pfu/well on 96-well plate. The studied innate markers determined via RT-qPCR were IFN-β, IFN-λ1 (IL-29), ISG54, MxA, MxB, TLR3, and IFN-κ. The data (mean±SD, N≥8) were normalized to GAPDH expression and presented as fold change to untreated cells, which were either uninfected (A) or infected (B). The values are colored based on their statistical difference to the unmodified siRNA swarm as per the table legend. P-values were determined by Mann-Whitney test. The relative sum of the statistical differences (SUM (p)) was calculated by adding or reducing 1 (p<0.05), 2 (p<0.01) or, 3 (p<0.001), depending whether the expression was significantly more or less, respectively, than that by the unmodified siRNA swarm.

More than unmodified				Less than unmodified			
p<0.001	p<0.01	p≤0.05	ns	ns	p≤0.05	0<0.01	p<0.001

A

No virus

	hpt	Unmodified	10% F-A	100% F-A	10% F-C	100% F-C	10% F-U	100% F-U	Nonspecific	Mock
IFN-β	6	7.2± 10.4	13.2± 24.9	4.7± 5.0	12.1± 16.2	51.6± 51.9	27.7± 18.8	34.8± 29.0	1.1± 2.6	6.7± 8.0
	24	5.8± 10.0	1.1± 1.0	1.1± 0.6	0.8± 0.9	2.3± 2.1	0.0± 0.9	1.0± 0.8	1.0± 1.1	3.1± 2.8
	48	0.9± 0.8	1.6± 1.0	1.8± 2.8	1.4± 1.3	25.2± 58.7	1.7± 1.5	2.5± 2.1	1.8± 2.7	1.0± 1.3
IL-29	6	9.7± 17.3	4.4± 6.0	2.0± 3.3	10.3± 16.0	49.0± 44.9	30.8± 36.2	45.8± 38.4	0.1± 0.2	1.3± 1.6
	24	7.8± 8.8	4.7± 3.7	1.8± 2.2	3.6± 2.2	18.8± 22.3	6.1± 5.4	9.6± 9.6	1.2± 1.6	6.2± 8.1
	48	5.2± 5.3	7.0± 7.5	3.4± 4.4	5.6± 5.1	61.0± 112.1	8.5± 8.0	6.4± 5.8	5.3± 8.7	6.5± 7.4
IFN-κ	6	4.1± 8.3	1.2± 2.4	0.3± 0.6	5.9± 11.7	0.4± 0.5	22.5± 5.5	0.0± 0.0	10.9± 21.7	1.5± 2.9
	24	0.2± 0.2	0.1± 0.1	0.7± 1.1	0.0± 0.1	0.2± 0.4	0.4± 0.7	0.2± 0.2	0.1± 0.1	1.0± 1.9
	48	0.4± 0.7	0.9± 1.0	0.2± 0.4	0.5± 1.0	0.0± 0.0	0.4± 0.5	1.0± 1.4	0.0± 0.0	0.0± 0.0
ISG54	6	51.2± 42.3	71.1± 19.1	24.5± 11.2	126.5± 96.0	177.9± 41.5	123.8± 42.1	148.2± 63.3	8.9± 2.0	10.7± 6.0
	24	15.9± 11.0	15.4± 9.9	15.1± 10.7	13.0± 5.5	25.2± 17.5	14.5± 8.1	23.8± 20.0	6.8± 4.9	23.0± 21.2
	48	9.2± 9.4	10.4± 9.3	11.2± 20.0	11.6± 10.5	53.8± 107.0	11.1± 9.0	11.7± 11.8	10.5± 12.4	11.6± 10.9
MxA	6	31.2± 29.4	50.6± 32.4	11.3± 6.6	39.3± 38.0	55.3± 25.6	68.5± 42.1	53.0± 24.0	6.4± 6.4	2.9± 2.9
	24	25.8± 15.4	22.5± 18.3	14.5± 10.0	21.8± 9.5	16.9± 8.9	19.5± 8.8	24.1± 13.5	10.9± 9.1	22.8± 16.1
	48	9.1± 6.9	9.6± 7.2	10.4± 17.1	7.5± 4.7	17.1± 26.8	8.1± 5.0	8.0± 3.9	8.3± 8.4	10.6± 6.0
MxB	6	236.7± 155.3	362.5± 246.8	43.5± 20.8	477.8± 210.3	382.1± 166.7	541.4± 223.8	379.8± 155.0	25.3± 22.5	5.5± 6.5
	24	22.3± 5.0	17.0± 5.7	10.7± 3.2	17.8± 3.0	14.4± 1.7	14.4± 3.8	18.0± 3.7	5.3± 3.4	15.0± 10.2
	48	2.2± 0.4	2.5± 1.0	1.5± 0.4	2.2± 0.1	2.8± 0.5	2.6± 1.2	3.3± 0.4	1.6± 0.4	3.5± 0.7
TLR3	6	2.3± 3.2	4.1± 7.6	1.3± 1.6	17.0± 24.1	6.5± 6.5	4.8± 5.9	5.5± 3.7	0.8± 0.8	1.0± 1.7
	24	11.1± 4.4	9.0± 5.8	8.4± 4.1	9.6± 4.9	12.2± 2.5	9.6± 2.9	12.5± 6.1	4.1± 2.3	12.3± 7.9
	48	8.1± 6.9	6.1± 4.8	9.0± 16.6	6.0± 3.9	20.5± 36.0	7.2± 4.4	7.3± 5.2	7.4± 7.4	8.0± 4.9
SUM (p)		NA	0	-7	2	10	3	7	-13	-5

B

With HSV

	hpt	Unmodified	10% F-A	100% F-A	10% F-C	100% F-C	10% F-U	100% F-U	Nonspecific	Mock
IFN-β	6	5.6± 8.5	32.2± 49.2	1.2± 2.8	18.5± 19.9	21.0± 23.6	23.1± 26.4	25.1± 39.1	0.1± 0.1	1.1± 2.1
	24	5.8± 6.4	4.5± 5.5	4.6± 4.2	8.6± 6.8	8.0± 6.6	7.2± 6.7	7.3± 5.2	8.6± 7.3	8.6± 10.9
	48	0.2± 0.2	0.9± 2.0	0.1± 0.1	0.1± 0.1	0.6± 0.5	0.5± 0.7	1.3± 3.1	3.2± 5.3	2.2± 2.7
IL-29	6	44.9± 56.1	65.8± 102.4	11.0± 23.4	124.5± 209.0	212.3± 212.3	141.1± 163.1	292.9± 272.0	0.0± 0.0	8.4± 16.3
	24	1.3± 1.2	2.2± 1.7	1.2± 0.8	2.3± 1.8	7.9± 5.4	2.8± 2.4	5.7± 3.5	1.1± 1.4	3.3± 3.8
	48	0.5± 0.5	0.7± 0.7	0.5± 0.4	0.6± 0.5	5.3± 4.8	0.9± 0.8	0.8± 1.0	3.5± 5.8	0.8± 2.0
IFN-κ	6	0.0± 0.0	0.9± 1.8	0.0± 0.0	0.0± 0.0	0.4± 0.9	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
	24	1.0± 1.3	1.5± 1.6	0.4± 0.4	21.4± 42.5	0.4± 0.8	4.0± 7.0	6.1± 7.0	9.8± 11.9	0.4± 0.7
	48	0.1± 0.1	0.0± 0.0	0.0± 0.1	0.1± 0.0	0.1± 0.2	0.1± 0.0	0.0± 0.0	1.7± 3.3	15.7± 27.2
ISG54	6	80.2± 69.4	118.6± 86.7	15.1± 8.5	78.6± 47.7	104.2± 37.0	167.4± 167.6	113.2± 67.9	6.5± 5.0	5.9± 5.3
	24	11.7± 10.5	13.2± 12.9	12.7± 8.4	11.1± 9.3	23.5± 14.7	13.9± 11.8	19.9± 15.6	12.0± 12.0	19.4± 21.7
	48	3.1± 1.9	3.2± 2.2	2.0± 1.3	3.9± 2.8	7.9± 5.4	3.3± 2.1	9.1± 9.5	9.9± 6.7	8.2± 4.3
MxA	6	42.5± 65.0	17.0± 11.4	24.0± 34.2	30.7± 17.3	35.4± 25.2	52.6± 62.7	31.8± 23.6	1.6± 2.7	0.3± 0.6
	24	9.9± 8.8	7.9± 6.4	8.3± 6.8	9.6± 6.2	10.6± 6.2	9.6± 7.1	12.0± 8.0	8.1± 7.7	6.7± 7.2
	48	4.9± 2.9	5.5± 4.4	3.4± 2.0	5.2± 4.7	5.2± 2.5	4.1± 3.0	7.0± 4.4	9.1± 6.7	11.0± 9.1
MxB	6	28.5± 24.4	19.2± 4.8	7.3± 5.7	64.3± 95.1	40.8± 13.3	13.2± 4.9	46.6± 27.4	7.0± 11.9	0.0± 0.0
	24	3.3± 1.2	3.0± 0.4	3.3± 0.7	3.6± 1.2	4.7± 1.1	3.4± 1.6	5.1± 2.6	1.2± 0.4	0.1± 0.1
	48	7.8± 1.6	5.2± 1.1	3.7± 0.7	5.1± 1.2	8.1± 2.5	6.9± 1.6	8.2± 1.1	13.4± 5.3	24.5± 15.3
TLR3	6	3.2± 4.1	4.3± 8.6	0.0± 0.0	7.1± 12.2	0.0± 0.0	6.7± 12.2	1.1± 1.2	1.7± 2.8	0.1± 0.2
	24	14.1± 15.1	18.2± 18.3	8.9± 5.5	12.2± 10.9	20.2± 17.2	15.2± 14.1	18.8± 15.9	10.7± 9.9	13.1± 14.1
	48	24.2± 19.8	17.0± 13.2	8.7± 5.4	33.1± 56.5	23.9± 10.6	23.6± 16.6	29.9± 30.7	15.2± 33.0	24.5± 40.2
SUM (p)		NA	1	-6	0	10	5	6	-6	-8

Table 7. The innate responses of HCE cells to **(A)** modified siRNA swarms and to **(B)** modified siRNA swarms and viral challenge. Treatments were done with 50 nM of the indicated RNA, and the infection with HSV-1-GFP at 4 hpt with 1000 pfu/well on 96-well plate. The studied innate markers determined via RT-qPCR were IFN-β, IFN-λ1 (IL-29), ISG54, MxA, MxB, TLR3, and IFN-κ. The data (mean±SD, N≥8) were normalized to GAPDH expression and presented as fold change to untreated cells, which were either uninfected (A) or infected (B). The values are colored based on their statistical difference to the unmodified siRNA swarm as per the table legend. P-values were determined by Mann-Whitney test. The relative sum of the statistical differences (SUM (p)) was calculated by adding or reducing 1 (p<0.05), 2 (p<0.01) or, 3 (p<0.001), depending whether the expression was significantly more or less, respectively, than that by the unmodified siRNA swarm.

A

No virus

	hpt	Unmodified	10% F-A	100% F-A	Nonspecific	Mock
IFN-β	6	2.8 ± 2.3	12.9 ± 9.5	2.8 ± 1.6	7.1 ± 5.1	1.7 ± 1.1
	24	7.3 ± 2.7	5.3 ± 3.8	5.9 ± 3.5	3.3 ± 2.0	2.0 ± 1.1
	48	22.9 ± 40.1	6.2 ± 6.5	21.5 ± 48.3	9.6 ± 14.8	10.7 ± 15.7
IL-29	6	51.2 ± 37.3	258.1 ± 203.8	76.6 ± 35.3	121.2 ± 84.8	10.4 ± 4.5
	24	23.7 ± 4.9	85.3 ± 27.8	63.5 ± 56.2	32.7 ± 18.3	10.1 ± 5.6
	48	32.5 ± 10.9	80.1 ± 52.3	50.7 ± 42.1	41.2 ± 15.8	38.6 ± 25.9
IFN-κ	6	1.3 ± 1.0	1.2 ± 1.0	1.5 ± 1.4	1.5 ± 1.2	1.6 ± 1.1
	24	0.3 ± 0.3	0.6 ± 0.7	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.2
	48	3.7 ± 5.4	4.3 ± 5.5	1.9 ± 1.4	2.2 ± 1.5	2.0 ± 1.5
ISG54	6	38.8 ± 25.7	123.6 ± 65.2	50.3 ± 17.6	75.1 ± 36.9	4.4 ± 1.3
	24	15.9 ± 3.0	26.4 ± 9.6	22.4 ± 16.2	24.8 ± 25.9	5.2 ± 1.5
	48	5.5 ± 1.1	9.4 ± 2.9	6.3 ± 3.5	5.9 ± 1.2	5.9 ± 2.3
MxA	6	7.2 ± 4.8	13.9 ± 5.8	7.5 ± 5.3	10.4 ± 4.2	1.6 ± 0.7
	24	9.3 ± 1.8	14.3 ± 4.5	11.6 ± 5.9	14.9 ± 11.0	5.8 ± 1.1
	48	6.2 ± 1.8	7.5 ± 2.4	5.4 ± 1.8	6.6 ± 1.8	8.1 ± 1.2
MxB	6	10.3 ± 4.0	33.6 ± 15.3	14.1 ± 3.9	10.9 ± 4.6	1.7 ± 0.9
	24	19.2 ± 13.3	25.4 ± 10.6	26.5 ± 21.3	28.85 ± 27.2	10.8 ± 6.9
	48	9.1 ± 2.7	12.1 ± 2.7	9.0 ± 5.3	10.9 ± 4.3	9.7 ± 4.1
TLR3	6	2.4 ± 1.9	9.5 ± 7.4	2.8 ± 1.9	1.6 ± 1.0	1.4 ± 1.2
	24	17.3 ± 6.7	30.2 ± 13.4	16.6 ± 10.0	10.4 ± 6.0	6.8 ± 2.8
	48	3.8 ± 2.4	5.1 ± 1.1	3.6 ± 2.7	2.8 ± 0.6	5.1 ± 3.2
SUM (p)		NA	22	0	0	-23

p<0.001	More than unmodified
p<0.01	
p<0.05	
ns	
ns	Less than unmodified
p<0.05	
0<0.01	
p<0.001	

B

With HSV

	hpt	Unmodified	10% F-A	100% F-A	Nonspecific	Mock
IFN-β	6	5.8 ± 7.5	13.3 ± 12.9	2.4 ± 1.1	6.2 ± 5.2	1.5 ± 1.7
	24	5.9 ± 3.5	7.3 ± 2.7	5.3 ± 3.8	3.3 ± 2.0	2.0 ± 2.0
	48	15.0 ± 23.6	14.6 ± 20.7	7.9 ± 10.0	17.5 ± 12.1	21.9 ± 31.4
IL-29	6	93.7 ± 120.5	184.0 ± 122.1	50.9 ± 14.6	109.4 ± 87.5	5.6 ± 4.2
	24	40.2 ± 19.7	77.6 ± 28.7	51.7 ± 44.1	33.4 ± 10.5	7.0 ± 3.9
	48	33.8 ± 22.4	81.4 ± 50.3	54.7 ± 59.1	56.0 ± 37.0	23.8 ± 30.9
IFN-κ	6	1.8 ± 1.8	1.3 ± 1.2	0.9 ± 0.7	1.4 ± 1.1	1.2 ± 0.6
	24	0.6 ± 0.6	0.4 ± 0.4	0.4 ± 0.4	0.4 ± 0.4	0.4 ± 0.5
	48	2.7 ± 2.7	2.4 ± 1.1	1.8 ± 0.9	8.2 ± 14.0	4.3 ± 4.8
ISG54	6	67.4 ± 63.6	133.2 ± 106.0	43.7 ± 10.9	75.1 ± 40.1	2.9 ± 0.7
	24	16.3 ± 6.4	22.2 ± 5.1	13.6 ± 7.3	16.3 ± 4.4	2.8 ± 1.6
	48	6.8 ± 3.0	10.4 ± 4.5	7.3 ± 5.5	5.6 ± 4.7	1.9 ± 1.2
MxA	6	9.9 ± 9.3	15.4 ± 11.0	6.9 ± 1.9	8.4 ± 4.7	1.2 ± 0.3
	24	18.1 ± 9.8	14.4 ± 5.4	9.7 ± 3.6	17.7 ± 3.7	6.5 ± 2.8
	48	10.5 ± 3.5	12.3 ± 5.8	7.6 ± 2.8	12.2 ± 13.1	3.6 ± 1.3
MxB	6	19.7 ± 19.9	33.5 ± 13.5	16.0 ± 5.3	19.0 ± 7.7	1.5 ± 1.3
	24	34.3 ± 9.5	22.3 ± 11.7	22.4 ± 17.2	31.8 ± 8.2	13.6 ± 5.9
	48	13.4 ± 4.7	16.4 ± 5.2	11.8 ± 6.5	10.9 ± 7.9	2.7 ± 2.0
TLR3	6	4.7 ± 4.5	8.2 ± 6.8	2.7 ± 1.6	1.9 ± 2.2	1.6 ± 1.2
	24	18.7 ± 7.4	33.5 ± 17.8	14.4 ± 6.0	16.0 ± 6.0	5.1 ± 2.0
	48	7.4 ± 7.6	6.7 ± 4.6	4.9 ± 5.2	1.9 ± 1.6	0.7 ± 1.3
SUM (p)		NA	7	-1	-1	-36

6 Discussion

6.1 From unmodified to 2′-fluoro-modified antiviral siRNA swarms

There are currently many hard-to-treat or untreatable viral infections lacking a vaccine, and thus in need of a novel treatment. Additionally, viral outbreaks lacking pre-existing treatment have been rather regular in the recent decades. And, as the recent COVID-19 (coronavirus disease 2019) pandemic has proven, despite the global capability for rapid vaccine development, there was and is a global need for an antiviral which tolerates viral variants. Such an antiviral could save millions of lives before, during, and after successful vaccine development. The development of antiviral siRNAs for such unmet medical needs is tempting, as viruses are always distinct from human cells by their transcripts, allowing for straightforward development of a safe antiviral, in principle. Moreover, the development of an antiviral siRNA requires basically only the information of the viral sequence, which is nowadays rapidly generated, if not already available. However, regular siRNAs have short and very limited target sequences, allowing for escape mutants.

The established enzymatic synthesis of siRNA swarms allows for rapid development of a pool of antiviral siRNAs, which can target even thousands of base pairs (bp) of viral sequence (Nygårdas et al., 2009, Romanovskaya et al., 2012). Therefore, an siRNA swarm, in contrast to a canonical 19-27 nucleotide (nt) long single site (ss) siRNA, circumvents the issue of escape mutants: as an siRNA swarm targets hundreds, or even thousands of base pairs, it is practically impossible for a virus to fully escape the treatment via mutation. The choice of target gene for the studies in this thesis was the UL29 gene of HSV-1, which is a β-gene essential in replication of the viral genome (Weir et al., 1989, Weir and Stow, 1990, Boehmer and Lehman, 1993, see also **Figure 2C**). The UL29 is a highly conserved gene (Bowen et al., 2019), and the chosen siRNA swarm target sequence has maximal homology between HSV-1 and HSV-2, and minimal homology to human and murine sequences (Paavilainen 2017). The UL29 siRNA swarm has been shown superior to UL54 and UL27 siRNA swarms, representing essential α- and γ-genes, respectively, when the innate immunity profiles and antiviral activities of the three anti-HSV siRNA swarms were compared (Paavilainen et al., 2015). Furthermore, prior to being

used as target to siRNA swarms, an UL29 siRNA was shown effective in an animal model (Palliser et al., 2006). Thus far, the UL29 targeted antiviral siRNA swarms have been shown efficient against HSV-1 *in vitro* in various studies (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016) and *in vivo* in a corneal infection model (Paavilainen et al., 2017). Furthermore, siRNA swarms have been shown antiviral also against influenza A virus (Jiang et al., 2019) and coxsackievirus B3 (Nygårdas et al., 2009) *in vitro*.

Though more wide-spectrum than regular antiviral siRNAs, antiviral siRNA swarms face challenges in their delivery and stability, as all RNA based drugs. The stability of the siRNAs may be enhanced by chemical modifications, popularly those in the 2′-position in the ribose moiety, such as 2′-OMe and 2′-fluoro modifications. These 2′-modifications have not only been shown to enhance stability in human serum (Choung et al., 2006), but also to subsequently increase bioavailability *in vivo* (Kenski et al., 2012), potency *in vitro* and *in vivo* (Allerson et al., 2005, Prakash et al., 2005, Muhonen et al., 2007, Foster et al., 2018), as well as to prolong the duration of action *in vitro*, particularly when the modifications are placed to nuclease sensitive sites (Muhonen et al., 2007, Volkov et al., 2009). Furthermore, the 2′-fluoro-modified nucleotides, especially those 2′-fluoro-adenosine modified (Fucini et al., 2012), should lead to reduction of innate response stimulation *in vitro* in contrast to the unmodified siRNAs. Importantly, all three siRNA medicines currently approved, patisiran (Onpattro®, EMA/554262/2018), givosiran (Givlaari®, EMA/CHMP/70703/2020), and lumasiran (Oxlumo®, EMA/568312/2020), have incorporated 2′-fluoro and 2′-OMe modifications, proving their safety in humans. For the aforementioned reasons, siRNA swarms were advanced by introducing 2′-modifications to their sequence. In study I, the enzymatic synthesis of 2′-fluoro-modified anti-HSV siRNA swarms with 2′-fluoro-adenosines, 2′-fluoro-cytidines, or 2′-fluoro-uridines was shown successful (study I Figure 1 and S3). The modified nucleotides were either partially (10%) or fully (100%) incorporated into the antisense strand, which was the superior strand in anti-picornavirus siRNAs (Schubert et al., 2007). The 2′-fluoro-guanosine and multi-2′-fluoro-modified nucleotides, as well as 2′-OMe nucleotides, were omitted due to complications in synthesis (study I Figure 1).

In siRNA swarm synthesis, where the long dsRNA is diced mainly to blunt ended 25 bp dsRNAs, the 2′-fluoro-modifications will be in differing locations in the individual siRNAs. Luckily, in contrast to the 2′-OMe modification, which may cause a shift in activity depending on its placement, siRNAs with 2′-fluoro-modifications have been shown to be well-tolerated by RISC and to have similar or superior activity to unmodified siRNAs, independent of the strand or position of the modification (Allerson et al., 2005, Prakash et al., 2005, Muhonen et al., 2007). Therefore, there is high potential for an siRNA swarm with 2′-fluoro-modifications

to be superior over an unmodified siRNA swarm. It is however unlikely that the successfully synthesized 2′-fluoro-adenosine, 2′-fluoro-cytidine, or 2′-fluoro-uridine-modified siRNA swarms would be equal to each other, as differently 2′-modified siRNAs usually require iterative optimizing to find the best candidate with enhanced potency and stability (Choung et al., 2006, Foster et al., 2018). Although the relatively low amount of modified nucleotides per the 2′-fluoro-modified UL29-siRNA swarm sequence (~17%, ~20%, or ~28% of the antisense strand of fully modified 2′-fluoro-A, 2′-fluoro-U, or 2′-fluoro-C siRNA swarms, respectively) could at first appear disadvantageous or inadequate over fully modifying a sequence, such as is done with givosiran and lumasiran, some literature actually suggests that lower relative amounts of 2′-fluoro-modified nucleotides in siRNAs are superior (Foster et al., 2018). Altogether, the previous literature supports finding an siRNA swarm with enhanced characteristics over an unmodified siRNA swarm from the successfully synthesized 2′-fluoro-modified siRNA swarms.

6.2 The 2′-fluoro-adenosine-modified siRNA swarms are well tolerated and highly potent (I, III)

In this thesis, anti-HSV UL29-targeted 2′-fluoro-modified siRNA swarms, with 2′-fluoro-adenosines, -cytidines, or -uridines partially (10%) or fully (100%) incorporated into the antisense strand were assessed *in vitro* for their cytotoxicity, stability, and antiviral efficacy, as well as for the effect they have on host responses, with or without abundant viral challenge. These *in vitro* studies were conducted in a proof of concept cell line, U373MG, allowing comparison with previous studies (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016). In parallel, a cell line representing the target tissue for herpes keratitis treatment, human corneal epithelial (HCE) cells, was used. The HCE cells are considered a highly translational cell line (Toropainen et al., 2001, Ranta et al., 2003, Toropainen et al., 2003, Rönkkö et al., 2016), which we validated for use in repetitive monolayer screening for antiviral RNAi studies against HSV-1 (study III Figure 3).

First, any cytotoxicity of the UL29-targeted anti-HSV 2′-fluoro-modified siRNA swarms was assessed at the concentration of 50 nM in U373MG and HCE cells **(Figure 4A)**. All the 2′-fluoro-modified siRNA swarms were well tolerated by U373MG cells, whereas in HCE cells, the 2′-fluoro-pyrimidine-modified siRNA swarms (10% F-C, 100% F-C and 10% F-U) were significantly less tolerated than mock transfection, and 100% F-C even decreased the relative viability to slightly below the acceptable 80% limit. The cytotoxicity was assessed also within a wider dose range of 0-150 nM (**Figure 4B**), where the viability profile was shown independent of the amount of modifications in the siRNA swarm. As the differences

detected in **Figure 4A** prevailed, any detected cytotoxicity is likely more dependent on the siRNA swarm and its modification, than on the concentration, at least in the studied dose range. The results are in line with previous research, where the dose-dependency of siRNA swarm concentration to cytotoxicity was studied until 500 nM with unmodified siRNA swarms in U373MG cells, but even then the viability was not significantly affected (Paavilainen, 2017). Therefore, although determination of the half-maximal cytotoxicity value (CC_{50}) would be of interest, it is likely not feasible with the currently available amounts of modified siRNA swarms, especially since the CC_{50} of 2′-fluoro-monomers is in µM scale (Janas et al., 2019). Nevertheless, it is evident that HCE cells are more sensitive to 2′-fluoro-modifications than U373MG cells, and especially to those in pyrimidines (**Figure 4A**). This result, combined with the elevated innate responses by the 100% F-C in U373MG cells (**Table 6**; I, Figure 4), and the lower antiviral efficacy by 10% F-U and 100% F-U (study I Figure 3B), led to considering the 2′-fluoro-adenosine-modified siRNA swarms as the best candidate for at least keratitis indications. Therefore, with HCE cells, the further studies were mainly conducted with 10% F-A and 100% F-A.

To study the antiviral efficacy of the 2′-fluoro-modified siRNA swarms, at first a similar setting as before (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016), with treatment preceding the infection by 4 hours, was used. The infection was kept at the same level as before, to best represent the clinical infection (Paavilainen, 2017). In both U373MG cells and HCE cells, all tested 2′-fluoro-modified siRNA swarms were antiviral, as they reduced virus originating GFP-signal (**Figure 5A**) as well as significantly decreased viral shedding (**Figure 5B, 5C**), and viral mRNA expression (**Figure 6**), including the expression of the RNAi target gene, UL29 (**Figure 6C, 6D**). The magnitudes of the efficacies in U373MG cells were even 100-times more than reported before (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016); here, the UL29-targeted siRNA swarms reached up to 100,000-fold (>99.999%) inhibition of viral shedding and more than 10,000-fold (>99.99%) inhibition of viral transcript expression in contrast to treatment with the nonspecific siRNA swarm. In HCE cells, the efficacies were not as high as in U373MG cells, but still at impressive levels for hard-to-transfect epithelial cells at roughly 100-fold (>99%) for both viral shedding and transcripts. In both cell types the efficacies of 2′-fluoro-modified and unmodified UL29-targeted siRNA swarms were similar, and did not significantly differ from each other (**Figure 5, 6**). However, in both cell types, and in both inhibition of viral shedding and of viral transcripts, there was a tendency for the modified siRNA swarms to be more antiviral than the unmodified siRNA swarms. This was especially apparent in the statistical significances determined against mock treatment, which were of higher or equal magnitude for all modified siRNA swarms, than for

unmodified siRNA swarms (**Figure 5B, 5C, 6G**). This finding is supported by the results of study I from U373MG cells, where with a higher amount of replicates almost all 2′-fluoro-modified siRNA swarms (10% F-A/C and 100% F-A/C/U) were shown statistically more antiviral than the unmodified siRNA swarm (study I Figure 3B). In HCE cells, statistical significance for increased antiviral efficacy was not reached, but the results are encouraging, and do suggest for enhanced gene silencing and antiviral efficacy with siRNA swarms harboring incorporated 2′-fluoro-modifications.

The dose-response of the concentration of 2′-fluoro-adenosine-modified siRNA swarms and unmodified siRNA swarms to inhibition of viral shedding was studied in both U373MG and HCE cells (**Figure 7**). The dose-responsiveness for both prophylactic and therapeutic efficacy was determined. Before, the dose-response relationship of siRNA swarm concentration to viral inhibition was not determined in such wide dose-range, nor has therapeutic efficacy with anti-HSV siRNA swarms been studied before *in vitro,* but actually only *in vivo* (Paavilainen et al., 2017). The therapeutic antiviral activity of an siRNA swarm is different on a cellular level to prophylactic activity, as in the therapeutic setting, the siRNA swarm will have an RNAi target immediately present, as the viral transcription is abundant already at a few hours post infection (e.g. study III Figure 2), and as the cell will not have abundant antiviral innate responses, which in prophylactic settings takes place immediately after transfection, in response to extracellular dsRNA (e.g. study I Figure 4). As an example, for the chimeric anti-influenza A virus siRNA swarm, there was no detectable therapeutic efficacy, although prophylactic efficacy was prevalent (Jiang et al., 2019). However, impressively, the anti-HSV UL29-targeted siRNA swarms, whether modified or not, had both prophylactic and therapeutic efficacies with nanomolar potencies (**Figure 7**). The IC_{50} values for HCE cells were slightly higher than those found from literature with different viruses and single site siRNA (Schubert et al., 2007, Moon et al., 2016), whereas the extrapolated IC_{50} values for U373MG cells were up to two magnitudes lower. The differences between the U373MG and HCE cell highlight the need to be aware of the treatment target cell types and tissues, which might require highly different amounts of siRNA to reach a similar level of efficacy. The dose we standardly used (study I, study III), 50 nM, was chosen based on preliminary results as it allowed maximum inhibitory efficacy with the unmodified siRNA swarm in both cell lines (**Figure 7**), with no cytotoxicity (**Figure 4**). However, retrospectively, 50nM is a rather extreme dose for U373MG cells. Despite the differences in the susceptibilities of the two cell lines to antiviral RNAi, the increased potency by modified siRNA swarms was evident in both cell lines. In U373MG cells, at doses below 2 nM, the modified siRNA swarms were more efficient than the unmodified siRNA swarms, especially in the therapeutic setting (**Figure 7A, 7B**). In HCE cells, the modified siRNA swarms were more

efficient throughout the dose-range (**Figure 7C, 7D**). However, in neither of the cell lines were the modified siRNA swarms statistically different to the unmodified siRNA swarms. Nevertheless, the IC_{50}-values of the modified siRNA swarms were constantly lower than those of the unmodified siRNA swarms (**Figure 7E**). This indicates a higher potency for siRNA swarms with 2′-fluoro-modifications, whether the treatment indication was therapeutic or prophylactic.

6.3 siRNA swarms retain full prophylactic and therapeutic activity for multiple days *in vitro* – with and without 2′-fluoro-modifications

In treatment of HSV-1 infections, the major unmet medical need is ACV-resistant herpes infection. It is common especially in patients in need of long-term prophylactic treatment, such as patients suffering from herpes keratitis. Therefore, most relevant future clinical use of the anti-HSV 2′-fluoro-modified siRNA swarms would be long-term topical, prophylactic treatment to prevent lytic infection by reactivating virus, and subsequent exacerbations. For such use, the siRNA swarm would need to have long-term prophylactic activity and stability. However, there is no prior knowledge on the duration of antiviral activity of siRNA swarms, as in prior assays conducted, the infection follows the treatment by four hours (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016). Therefore, the duration of efficacy of antiviral unmodified and 2′-fluoro-modified siRNA swarms was studied in U373MG and HCE cells. For the study, 10% and 100% F-C modified siRNA swarms were used, as in study I, the 100% F-C was shown to be resistant to RNase A (study I Figure 1C) and to have antiviral efficacy well beyond the unmodified siRNA swarm (study I Figure 3C). Therefore, detecting a difference between modified and unmodified siRNA swarms would be likely with 10% or 100% F-C, if there is any to be detected. The prolonged efficacy was studied for four days (**Figure 8**), by treating the cells at day 0, and infecting them at 4, 24, 48, 72, or 96 hours post treatment. In U373MG cells, the siRNA swarms remained stable and equally active throughout the experiment (**Figure 8A**). In HCE cells, the antiviral siRNA swarms were not as active on day 4 as on the previous days, with at least a 30% decrease in inhibition of viral shedding, independent of the modifications (**Figure 8B**). Interestingly, the inhibition of viral shedding was not decreased after the earliest timepoints, even though the antiviral host innate responses of both cell types to extracellular dsRNA are most elevated on the day of and on the first day after the infection (study I Figure 4; study III Figure 3). Moreover, as opposed to the hypothesis, in this assay, the modified siRNA swarms did not remain active longer than unmodified siRNA swarms, even though especially in HCE cells, such effect would have been possible to be detected in the latest timepoint (**Figure 8**). However,

a major factor in siRNAs retaining their activity, is the division rate of the cells (Bartlett and Davis, 2006), which was a constant for all different siRNA swarms in this assay. Therefore, based on these results, any increase in stability by the incorporated 2′-fluoro-modifications cannot be excluded. The results rather suggest that the phenomenon cannot be studied with such simple settings *in vitro*, but would require for example non-dividing cells, or *in vivo* studies. Nevertheless, the siRNA swarms did retain their full activity for at least three days after treatment. The result is in line with literature, where single site siRNAs are reported to start losing their *in vitro* activity at 4 days, and finally to lose all *in vitro* activity within one week (Bartlett and Davis, 2006). Astonishingly, one siRNA swarm dose could control the viral spread fully, for at least 4 days after the infection, when the treatment was given therapeutically (**Figure 9**, grey bars). In controlling the viral spread, within the limits of the assay, the modified siRNA swarms were equal to the unmodified siRNA swarms.

Despite promising *in vitro* data, it would not be likely that any drug would allow immediate, total protection against HSV-1. In the treated tissue, there will be cells in which the lytic replication cycle will be able to initiate, and there will be progeny viruses spreading to the neighboring cells. In this study, the phenomenon is seen with the prophylactic inhibition of viral transcripts, which is extreme, but never total (**Figure 6, Figure 7**). In principle, independent of the future indication, the siRNA swarms would in a true clinical situation act prophylactically and therapeutically within the same tissue. Basically, the infected tissue would have healthy cells, to which the virus is aiming to spread, in need of prophylactic protection, as well as infected cells, in need of a therapeutic antiviral activity. There would also be treated cells already clearing the lytic infection, facing a repeated viral challenge, as well as cells needing a repeated dose, to enhance the antiviral activity of the existing siRNA, or as the first prophylactic or therapeutic treatment. For these reasons, and as the prophylactic and therapeutic efficacies were evident (**Figure 7, Figure 8**), the antiviral efficacy of antiviral siRNA swarms was of interest, both under repeated viral challenge and after a repeated dose of the antiviral siRNA swarms. In the used setting, the U373MG and HCE cells were first infected, treated at 4 hpi, and then at 2 dpi left as they were, retreated, or re-challenged with viral infection (**Figure 9**). The repeated treatment did not increase the inhibition of viral shedding in either cell line with any of the treatments, which was unsurprising, as there was basically no room for improvement, since in the canonically treated cells the inhibition of viral shedding was close to 100% (**Figure 9**, grey bars vs white bars). Therefore, the repeated dosing requires additional research, for example with extended timeframes or decreased siRNA concentrations. In U373MG cells, all treatments were able to diminish the viral rechallenge without any sign of decreased inhibition efficacy (**Figure 9A**), however, in HCE cells, the fully 2′-fluoro-modified siRNA swarm

(100% F-C) was significantly more able to control the repeated viral challenge than the unmodified siRNA swarm (**Figure 9B**). Therefore, it is possible that incorporated modifications increase the endurance of antiviral siRNA swarms, as is supported by the increased potency by 2′-fluoro-modified siRNA swarms (**Figure 7**). Nevertheless, the claim for increased endurance would require confirmation by more research, as would the possibility for prolonged stability by incorporated 2′-fluoro-modifications.

Most likely the improvements by the 2′-fluoro modifications are not reflected to the full extent in monolayer cell cultures, such as were used in this thesis. The obvious advantages of the 2′-fluoro modifications arise from their nuclease resistance, which increases the half-life and bioavailability of the siRNAs. Likely, after the siRNAs have entered the cell and are loaded by the RISC complex, the functional half-lives of modified and unmodified siRNA become equal. This could dilute the differences between the 2′-fluoro-modified and modified siRNAs in the assays of this thesis. Therefore, *in vivo* models or even three dimensional (3D) cell culture models could better demonstrate any enhanced stability, efficacy, or potency of the 2'-fluoro-modified siRNA swarms. Nevertheless, the conducted *in vitro* studies in monolayer cell cultures were an important prerequisite for beginning this avenue of research, and, though used as monolayer, the HCE cell line provides relatively high translationality for the results. Altogether, the results gathered on infection prevention and control are promising to the extent that continuation directly into a series of *in vivo* studies should not be excluded.

6.4 Modified and unmodified siRNA swarms are highly efficient against clinical isolates, despite their varying sensitivity to acyclovir (II)

One of the fundamental milestones in the preclinical development of siRNA swarms for treatment of HSV-1 is to show that clinical isolates with varying sensitivity to ACV are susceptible to siRNA swarm treatment. Therefore, the efficacy of unmodified and 2′-fluoro-modified siRNA swarms was studied against clinical HSV-1 strains which had a varying sensitivity to ACV (study II Figure 1, (Kalke et al., 2022)). All the clinical isolates included (N=53) were sensitive to ACV. The ACV sensitivity varied from to 0.08 to 0.43 µg/ml for the strains used with unmodified siRNA swarms in Figure 10 (study II), and from 0.14 to 1.13 µg/ml, for the strains used with 2′-fluoro-modified siRNA swarms in Figure 11 (Kalke et al., 2022). The unmodified siRNA swarms were highly effective against all tested clinical strains with only minimal variation in their sensitivity and no outliers **(Figure 10).** Although the mechanisms of action of ACV and of the UL29-targeted siRNA swarm are different and target different viral proteins, it is important to

emphasize that the sensitivities to ACV and to unmodified siRNA swarm did not correlate, but were independent from each other (study II Figure 3B). The result highlights that in clinical use, if an infection would start showing decreased sensitivity to ACV, an UL29-siRNA swarm would be a feasible treatment option, as simultaneous decreased sensitivity to an UL29-siRNA swarm would not be expected. The efficacy of the unmodified siRNA swarm against ACV-resistant HSV-1 was confirmed using a thymidine kinase deficient strain, Δ305. The ACV-resistant Δ305 was as sensitive to the siRNA swarm as the reference strain 17+, whose sequence was used as a template for siRNA swarm synthesis (study II Figure 2). In other words, Δ305 showed no decrease in sensitivity to the UL29-targeted siRNA swarm, despite its extreme ACV resistance (**Figure 10**). Furthermore, the minor variation detected in the UL29 sequences of the clinical isolates did not have any effect in the determined UL29-siRNA swarm sensitivity (study II Table 3 and Figure S3), confirming the siRNA swarm concept as an approach to overcome viral escape mutants in therapeutic use.

As with the unmodified siRNA swarm, the 2′-fluoro-modified siRNA swarm (100% F-A) was also highly efficient against all tested circulating strains, with inhibition of viral shedding of at least 99.8% in U373MG cells (**Figure 11**). In contrast, though not directly comparable, the maximum inhibitory efficacy of the unmodified siRNA swarm in Vero cells ranged from 97 to 100% (study II Table 3). Therefore, both unmodified and modified siRNA swarms are highly efficient against clinical isolates despite their varying ACV sensitivity. Additionally, the results show that the reference strains 17+, KOS, and F, are translational to clinical isolates in UL29-targeted RNAi studies, at least *in vitro*, as they were susceptible to siRNA swarms on similar levels as the clinical strains (**Figure 10, Figure 11**).

The set of isolates studied with the modified siRNA swarms were shown diverse also in respect to their replication characteristics. For example, the isolates which were slightly less susceptible to the 100% F-A antiviral siRNA swarm, V7, V12, V24, and V27 (**Figure 11**), were identified as having a rapid replication cycle (V7, V12) or as being highly cytotoxic (V27) in U373MG cells (Kalke et al., 2022). Regardless of these stand-out phenotypes, the 2′-fluoro-modified siRNA swarms were nevertheless able to eradicate them almost completely, which, once more, shows high promise to siRNA swarm treatment for therapeutic use.

Notably, in the assay for the efficacy of the 2′-fluoro-modified UL29-targeted siRNA swarm (100% F-A) against clinical isolates (**Figure 11A**), for the first and only time thus far, a 2′-fluoro-modified control siRNA swarm (100% F-A nonspecific) was used. Therefore, the gene-specific silencing efficacy of the 100% F-A siRNA swarm is now shown with even more confidence than of any other of the modified siRNA swarms.

6.5 The fully 2′-fluoro-adenosine-modified siRNA swarm has a favorable innate response profile (I, III)

The host innate responses to siRNA swarms, with or without a viral challenge present, were studied at 8, 24, and 48 hpt in U373MG (**Table 6**) and in HCE cells (**Table 7**). The markers studied were interferon beta (IFN-β), interferon lambda 1 (IFN-λ1; IL-29), interferon stimulated gene 54 (ISG54), human myxovirus resistance protein A (MxA), human myxovirus resistance protein B (MxB), toll-like receptor 3 (TLR3) and interferon kappa (IFN-κ). The panel included the type I and type III interferons, IFN- β and IFN-λ1, respectively, as well as TLR3 and ISG54, which have been shown valuable at differentiating between different types of antiviral siRNA (Paavilainen et al., 2015, Paavilainen et al., 2016). Before, in epithelial, neuronal, and retinal cells the responses to *Giardia* dicer cleaved siRNA swarms, also known as unmodified siRNA swarms, were shown to be minimal, but upregulation of especially type I and III interferons and TLR3 were prominent (Paavilainen et al., 2015, Paavilainen et al., 2016). In this thesis, also MxA, MxB, and IFN-κ were included as novel markers to study with antiviral siRNA swarms in the panel of innate responses. Similarly to IFN- β, IFN-λ1, TLR-3, and ISG54, they have been shown to be a part of antiviral cellular responses against HSV-1 (Ku et al., 2011, Crameri et al., 2018, Li et al., 2020). Therefore, if an siRNA swarm would induce extreme levels of one or more of the innate markers in the panel, it could not be considered highly gene specific. For example, the cytotoxic 88 bp dsRNA is highly antiviral, despite lacking any sequence specific activity, due to elevated innate responses and cytotoxicity (study III Figure 3B, S2, and S3). Throughout the panel of studied innate markers, the responses of neuronal (U373MG) and corneal epithelial (HCE) cells to modified siRNA swarms have been minimal, as they have been similar to those towards unmodified siRNA swarms, or to those induced by the transfection reagent alone (**Table 6, Table 7**; study I Figure 4; study III Figure 4). Therefore, all modified siRNA swarms may be considered sequence specific for their antiviral efficacy.

Despite the minimal induction of innate responses, as before, each studied marker was upregulated by transfection with the antiviral siRNA swarms (**Table 6, Table 7**). The upregulation was at similar level, whether the cells were challenged with virus or not. Therefore, at least in monolayer cell culture, during prophylactic antiviral siRNA treatment, the incoming virus has little effect to the cellular innate responses already elevated by the RNA treatment. However, notably, the responses to virus alone are mostly similar to those of untreated cells (study III Figure 3A). HSV-1 is likely downregulating a majority or all of the studied the innate responses, directly or indirectly, which leads to their elevation only at the later time points, when the infection is most abundant, and even to significant downregulation of

some, such as TLR3 (study III Figure 3A; (Peri et al., 2008)). Despite the minimal differences between the innate responses of infected and uninfected treated cell cultures, checking them is vital to ensure that there is no adverse mounting of innate responses.

Although the innate responses by the siRNA swarms were minimal, there were differences between the swarms used: in U373MG cells, where all the modified siRNA swarms (10% F-A/C/U, 100% F-A/C/U) were studied, the 100% F-C was significantly more immunostimulatory than the unmodified siRNA swarm, and the 100% F-A had the tendency of being the least immunostimulatory, together with the unmodified siRNA swarm (study I Figure 4). The 100% F-A had the tendency of being the least immunostimulatory also in HCE cells (study III Figure 4). This result was repeated in the analysis of Tables 6 and 7, where in both cell lines, the 100% F-A was clearly the least immunostimulatory of the antiviral treatments tested. By the relative p-value sums (sum(p)) covering all studied innate response markers, in U373MG cells, the 100% F-A evoked even less immune responses than unmodified siRNA swarm (**Table 6**), whereas in HCE cells, 100% F-A was similar to unmodified siRNA swarm (**Table 7**). Interestingly, the 10% F-A did not show characteristics somewhere between the unmodified and the 100% F-A, but was more immunostimulatory than the unmodified siRNA swarm in both cell lines (**Table 6, Table 7**).

The reduced immunostimulation by incorporation of 2′-fluoro-adenosines is supported by literature (Fucini et al., 2012). Such minimal changes in innate response are preferred, as it is both a sign of and leads to minimal off-target effects, or unexpected adverse events. Furthermore, with minimal effect on innate responses, the efficacy of the antiviral siRNA is more likely to be gene specific. For treatment safety, at least in keratitis indications, the innate responses to the siRNA should be minimal, as the eye is already challenged by pathologic inflammation due to the recurrent infections. Nevertheless, interferons induced by the dsRNA are antiviral, and could thus in some antiviral indications be preferred. For such scenarios, the immunostimulatory 100% F-C, which is also gene specific and highly antiviral (study I Figure 3B), could be the priority candidate. Although, for 100% F-C, *in vitro* studies with a 100% F-C modified nonspecific control would be required, to find out in contrast to other siRNA swarms (study I Figure 3B), to what extent its elevated antiviral efficacy is gene specific. However, for keratitis indications, if external support to the innate antiviral control would be preferred, concomitant treatment with interferons, though debated (Minkovitz and Pepose, 1995, Schlee et al., 2006, Nguyen et al., 2009, Wilhelmus, 2015), might be more feasible than using an immunostimulatory siRNA.

7 Summary and conclusions

In treatment of HSV-1 infections, the major unmet medical need to be tackled is ACV-resistant herpes infection, which is especially common in patients in need of a long-term prophylactic treatment, such as patients suffering from herpes keratitis. siRNA swarms offer an option for antiviral treatment with a high tolerance for pathogen diversity, in contrast to regular siRNA. Previously, siRNA swarms have been shown to be well tolerated and to induce only minimal innate responses *in vitro*, as well as to have antiviral efficacy against multiple reference and clinical strains of HSV-1 *in vitro*, with no emerging resistance over multiple passages of treatment, and antiviral efficacy *in vivo* in a model of corneal infection (Romanovskaya et al., 2012, Paavilainen et al., 2015, Paavilainen et al., 2016, Paavilainen et al., 2017). Despite the promising results, siRNA swarms face challenges with stability, as all RNA drugs. Therefore, in this study, UL29-targeted siRNA swarms with incorporated 2′-fluoro-modifications, supposed to increase the stability and potency of the RNA, were validated *in vitro*. The modified siRNA swarms harbored 2′-fluoro-modifications in either all or a part of their cytidine, uridine, or adenosine residues. In cells representing the nervous system (U373MG), all of the modifications were well tolerated (**Figure 4**), with minimal innate responses (study III Figure 4), and antiviral efficacies beyond those of the unmodified counterpart (study III Figure 3). However, in a human corneal epithelial cell line, considered highly translational for keratitis research and self-validated for use in anti-HSV-1 RNAi studies (study II Figure 3), the 2'-fluoro-adenosine modified siRNA swarms were clearly the best candidates, as the pyrimidine modified siRNA swarms were not as well tolerated (**Figure 4**). Even though best tolerated, the 2′-adenosine-modified siRNA swarms are by no means least aggressive against HSV-1 infection: in addition to the significant and extreme antiviral efficacy with up to 100,000-fold reduction of viral shedding (**Figure 5**) and 10,000-fold reduction of viral transcripts (**Figure 6**), the 2'-fluoro-adenosine modified siRNA swarms were shown to be more potent than unmodified siRNA swarms in both therapeutic and prophylactic assays (**Figure 7**), promising for comprehensive antiviral efficacy *in vivo*. Moreover, according to the overall profile of the studied innate responses, the 100% 2′-fluoro-adenosine modified siRNA swarm (100% F-A) was shown least immunostimulatory

of all the antiviral siRNA swarms tested in both U373MG and in HCE cells (**Table 6, Table 7**). Naturally, the minimal innate immunity induction is preferred, in order to avoid any unexpected adverse inflammation in therapeutic use.

After promising results of increased prophylactic and therapeutic potency by incorporation of the modifications (**Figure 7**), the stability of the modified siRNA swarms was studied. The results were surprising, as both modified and unmodified siRNA swarms were shown to retain full prophylactic activity for 3-4 days (**Figure 8**), depending on the cell type, and to control HSV-1 infection for at least 4 days with one therapeutic dose (**Figure 9**). However, reflecting the demonstrated increase in potency, under repeated viral challenge, the modified siRNA swarms were more potent (**Figure 9**). As the antiviral activity of all the UL29-targeted siRNA swarms was high for multiple days, the results encourage continuation to *in vivo* studies to reveal any increase in stability by the incorporated modifications.

Never before was the efficacy of the siRNA swarms directly compared to that of the first-line treatment, ACV. Therefore, a total of 56 clinical isolates with known or determined ACV sensitivities were studied for their susceptibility to antiviral modified or unmodified siRNA swarms. Without exception, the clinical isolates were highly sensitive to the siRNA swarm treatment with minimal variation, despite their varying sensitivity to ACV (**Figure 10, Figure 11**). Furthermore, an ACV-resistant, thymidine kinase deficient strain was shown highly susceptible to siRNA swarm treatment (**Figure 10**). The results confirm the feasibility of siRNA swarm treatment for the unmet medical need that is ACV-resistant infection.

As a conclusion, the results of this thesis add new, highly promising data to the already comprehensive knowledge on the broad efficacy of antiviral siRNA swarms. The results demonstrate their minimal innate immunity response inductions in yet another cell line as well as their prophylactic and therapeutic antiviral efficacies, which last for multiple days without any reduction in efficacy, even in the presence of a repeated viral challenge. Most importantly, their efficacy against an ACV-resistant HSV-1 was shown. Moreover, a new type of siRNA swarm with incorporated 2′-fluoro-modifications was validated. The results demonstrate the superiority of siRNA swarms with incorporated 2′-fluoro-modifications over unmodified siRNA swarms, and raise the fully 2'-fluoro-adenosine modified UL29-targeted siRNA swarm (100% F-A) as the top candidate for further studies for antiviral siRNA development. The 100% F-A was the least immunostimulatory of the modified siRNA swarms, even less so than the unmodified siRNA swarm, meanwhile having elevated antiviral efficacy and more potency than the unmodified siRNA swarm against HSV-1 *in vitro*. However, any increased stability is left to be determined. Nevertheless, the accumulating promising results demonstrate the potential of siRNA swarms, whether modified or not, for treatment of ACV-resistant herpes infection, especially that of the eye. Furthermore, the 2′-fluoro-modified

siRNA swarm approach is feasible also from a regulatory point of view, as the safety of 2′-fluoro-modifications in humans is already proven by the three approved siRNA drugs, and, though currently withdrawn, an antiviral oligonucleotide, fomivirsen, was effective and safe in antiviral therapy of the eye. The only novelty for the authorities is the siRNA swarm in itself, which is different in its heterogeneity from a pool of single site siRNAs. (Also in this respect the 100% F-A should be preferred over the 10% F-A, as it is more homogenous.) However, not all drugs are homogenous, as for example oncolytic viruses and cell therapies, approved by both EMA and FDA, are rather heterogeneous as therapy products. Therefore, the heterogeneity of siRNA swarms should not be an insurmountable challenge.

Altogether, the results and current landscape encourage taking the next step in the preclinical pipeline of anti-HSV siRNA swarms, which should be an *in vivo* experiment confirming the potency and stability of 100% F-A, or a series of experiments to find solution to the next hurdle of RNA drugs: delivery. Nevertheless, nothing is excluding the possibilities of using the profound expertise and knowledge gathered in the anti-HSV work to tackle any other unmet medical need. For example, treatment of emerging or hard-to-treat pathogens could benefit from a well-tolerated, highly potent, rapidly synthesizable mediator of antiviral RNAi with high tolerance for pathogen variation – such as modified siRNA swarms.

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References

Aalto, A. P., Sarin, L. P., Van Dijk, A. A., Saarma, M., Poranen, M. M., Arumae, U. & Bamford, D. H. 2007. Large-Scale Production of dsRNA and siRNA Pools for RNA Interference Utilizing Bacteriophage Phi6 RNA-Dependent RNA Polymerase. *RNA,* 13**,** 422-9.

Ablashi, D., Agut, H., Alvarez-Lafuente, R., Clark, D. A., Dewhurst, S., Diluca, D., Flamand, L., Frenkel, N., Gallo, R., Gompels, U. A., Hollsberg, P., Jacobson, S., Luppi, M., Lusso, P., Malnati, M., Medveczky, P., Mori, Y., Pellett, P. E., Pritchett, J. C., Yamanishi, K. & Yoshikawa, T. 2014. Classification of HHV-6A and HHV-6B as Distinct Viruses. *Arch Virol,* 159**,** 863-70.

Adamiak, B., Trybala, E., Mardberg, K., Johansson, M., Liljeqvist, J. A., Olofsson, S., Grabowska, A., Bienkowska-Szewczyk, K., Szewczyk, B. & Bergstrom, T. 2010. Human Antibodies to Herpes Simplex Virus Type 1 Glycoprotein C Are Neutralizing and Target the Heparan Sulfate-Binding Domain. *Virology*, 400, 197-206.

Allerson, C. R., Sioufi, N., Jarres, R., Prakash, T. P., Naik, N., Berdeja, A., Wanders, L., Griffey, R. H., Swayze, E. E. & Bhat, B. 2005. Fully 2'-Modified Oligonucleotide Duplexes with Improved In Vitro Potency and Stability Compared to Unmodified Small Interfering RNA. *J Med Chem,* 48**,** 901-4.

Amjadi, O., Rafiei, A., Haghshenas, M., Navaei, R. A., Valadan, R., Hosseini-Khah, Z., Omran, A. H., Arabi, M., Shakib, R. J., Mousavi, T. & ashrafi, G. H. 2017. A Systematic Review and Meta-Analysis of Seroprevalence of Varicella Zoster Virus: a Nationwide Population-Based Study. *J Clin Virol,* 87**,** 49-59.

Arain, N., Paravastu, S. C. & Arain, M. A. 2015. Effectiveness of Topical Corticosteroids in Addition to Antiviral Therapy in the Management of Recurrent Herpes Labialis: a Systematic Review and Meta-Analysis. *Bmc Infect Dis,* 15**,** 82.

Arbuckle, J. H., Gardina, P. J., Gordon, D. N., Hickman, H. D., Yewdell, J. W., Pierson, T. C., Myers, T. G. & Kristie, T. M. 2017. Inhibitors of the Histone Methyltransferases Ezh2/1 Induce a Potent Antiviral State and Suppress Infection by Diverse Viral Pathogens. *Mbio,* 8.

Aubert, M., Strongin, D. E., Roychoudhury, P., Loprieno, M. A., Haick, A. K., Klouser, L. M., Stensland, L., Huang, M. L., Makhsous, N., Tait, A., De Silva Feelixge, H. S., Galetto, R., Duchateau, P., Greninger, A. L., Stone, D. & Jerome, K. R. 2020. Gene Editing and Elimination of Latent Herpes Simplex Virus In Vivo. *Nat Commun,* 11**,** 4148.

Awasthi, S. & Friedman, H. M. 2022. an mRNA Vaccine to Prevent Genital Herpes. *Transl Res,* 242**,** 56-65.

Awasthi, S., Hook, L. M., Pardi, N., Wang, F., Myles, A., Cancro, M. P., Cohen, G. H., Weissman, D. & Friedman, H. M. 2019. Nucleoside-Modified mRNA Encoding HSV-2 Glycoproteins C, D, and E Prevents Clinical and Subclinical Genital Herpes. *Sci Immunol,* 4.

Bartlett, D. W. & Davis, M. E. 2006. Insights into the Kinetics of siRNA-Mediated Gene Silencing From Live-Cell and Live-Animal Bioluminescent Imaging. *Nucleic Acids Res,* 34**,** 322-33.

Barton, S. E., Davis, J. M., Moss, V. W., Tyms, A. S. & Munday, P. E. 1987. Asymptomatic Shedding and Subsequent Transmission of Genital Herpes Simplex Virus. *Genitourin Med,* 63**,** 102-5.

Belshe, R. B., Leone, P. A., Bernstein, D. I., Wald, A., Levin, M. J., Stapleton, J. T., Gorfinkel, I., Morrow, R. L., Ewell, M. G., Stokes-Riner, A., Dubin, G., Heineman, T. C., Schulte, J. M., Deal,

C. D. & Women, H. T. F. 2012. Efficacy Results of a Trial of a Herpes Simplex Vaccine. *N Engl J Med,* 366**,** 34-43.

Bergmann, M., Beer, R., Kofler, M., Helbok, R., Pfausler, B. & Schmutzhard, E. 2017. Acyclovir Resistance in Herpes Simplex Virus Type I Encephalitis: A Case Report. *J Neurovirol,* 23**,** 335-337.

Bermek, O. & Williams, R. S. 2021. the Three-Component Helicase/Primase Complex of Herpes Simplex Virus-1. *Open Biol,* 11**,** 210011.

Biswas, S., Miguel, R. N., Sukla, S. & Field, H. J. 2009. A Mutation in Helicase Motif Iv of Herpes Simplex Virus Type 1 UL5 That Results in Reduced Growth in Vitro and Lower Virulence in a Murine Infection Model is Related to the Predicted Helicase Structure. *J Gen Virol,* 90**,** 1937-1942.

Biswas, S., Smith, C. & Field, H. J. 2007a. Detection of HSV-1 Variants Highly Resistant to the Helicase-Primase Inhibitor Bay 57-1293 at High Frequency in 2 of 10 Recent Clinical Isolates of HSV-1. *J Antimicrob Chemother,* 60**,** 274-9.

Biswas, S., Swift, M. & Field, H. J. 2007b. High Frequency of Spontaneous Helicase-Primase Inhibitor (Bay 57-1293) Drug-Resistant Variants in Certain Laboratory Isolates of HSV-1. *Antivir Chem Chemother,* 18**,** 13-23.

Biswas, S., Tiley, L. S., Zimmermann, H., Birkmann, A. & Field, H. J. 2008. Mutations Close to Functional Motif IV in HSV-1 UL5 Helicase That Confer Resistance to HSV Helicase-Primase Inhibitors, Variously Affect Virus Growth Rate and Pathogenicity. *Antiviral Res,* 80**,** 81-5.

Blot, N., Schneider, P., Young, P., Janvresse, C., Dehesdin, D., Tron, P. & Vannier, J. P. 2000. Treatment of an Acyclovir and Foscarnet-Resistant Herpes Simplex Virus Infection with Cidofovir in a Child After an Unrelated Bone Marrow Transplant. *Bone Marrow Transplant,* 26**,** 903-5.

Boehmer, P. E. & Lehman, I. R. 1993. Herpes Simplex Virus Type 1 ICP8: Helix-Destabilizing Properties. *J Virol,* 67**,** 711-5.

Bollaerts, K., Riera-Montes, M., Heininger, U., Hens, N., Souverain, A., Verstraeten, T. & Hartwig, S. 2017. A Systematic Review of Varicella Seroprevalence in European Countries Before Universal Childhood Immunization: Deriving incidence From Seroprevalence Data. *Epidemiol Infect,* 145**,** 2666-2677.

Bowen, C. D., Paavilainen, H., Renner, D. W., Palomäki, J., Lehtinen, J., Vuorinen, T., Norberg, P., Hukkanen, V. & Szpara, M. L. 2019. Comparison of Herpes Simplex Virus 1 Strains Circulating in Finland Demonstrates the Uncoupling of Whole-Genome Relatedness and Phenotypic Outcomes of Viral Infection. *J Virol,* 93.

Broberg, E. K., Nygårdas, M., Salmi, A. A. & Hukkanen, V. 2003. Low Copy Number Detection of Herpes Simplex Virus Type 1 mRNA and Mouse Th1 Type Cytokine mRNAs by Light Cycler Quantitative Real-Time PCR. *J Virol Methods,* 112**,** 53-65.

Brown, Z. A., Wald, A., Morrow, R. A., Selke, S., Zeh, J. & Corey, L. 2003. Effect of Serologic Status and Cesarean Delivery on Transmission Rates of Herpes Simplex Virus from Mother to Infant. *Jama,* 289**,** 203-9.

Burn, C., Ramsey, N., Garforth, S. J., Almo, S., Jacobs, W. R., Jr. & Herold, B. C. 2018. A Herpes Simplex Virus (HSV)-2 Single-Cycle Candidate Vaccine Deleted in Glycoprotein D Protects Male Mice From Lethal Skin Challenge with Clinical Isolates of HSV-1 and HSV-2. *J Infect Dis,* 217**,** 754-758.

Burns, W. H., Saral, R., Santos, G. W., Laskin, O. L., Lietman, P. S., Mclaren, C. & Barry, D. W. 1982. Isolation and Characterisation of Resistant Herpes Simplex Virus After Acyclovir Therapy. *Lancet,* 1**,** 421-3.

Campadelli-Fiume, G., Amasio, M., Avitabile, E., Cerretani, A., forghieri, C., Gianni, T. & Menotti, L. 2007. The Multipartite System that Mediates Entry of Herpes Simplex Virus into the Cell. *Rev Med Virol,* 17**,** 313-26.

Cerutti, H. & Casas-Mollano, J. A. 2006. On the Origin and Functions of RNA-Mediated Silencing: From Protists to Man. *Curr Genet,* 50**,** 81-99.

Chakrabarti, S., Pillay, D., Ratcliffe, D., Cane, P. A., Collingham, K. E. & Milligan, D. W. 2000. Resistance to Antiviral Drugs in Herpes Simplex Virus Infections Among Allogeneic Stem Cell Transplant Recipients: Risk Factors and Prognostic Significance. *J Infect Dis,* 181**,** 2055-8.

Challberg, M. D. 1986. A Method for Identifying the Viral Genes Required for Herpesvirus DNA Replication. *Proc Natl Acad Sci U S A,* 83**,** 9094-8.

Chen, Y., Bai, P., Mackay, S., Korza, G., Carson, J. H., Kuchta, R. D. & Weller, S. K. 2011. Herpes Simplex Virus Type 1 Helicase-Primase: DNA Binding and Consequent Protein Oligomerization and Primase Activation. *J Virol,* 85**,** 968-78.

Chono, K., Katsumata, K., Kontani, T., Shiraki, K. & Suzuki, H. 2012. Characterization of Virus Strains Resistant to the Herpes Virus Helicase-Primase Inhibitor ASP2151 (Amenamevir). *Biochem Pharmacol,* 84**,** 459-67.

Chou, J. & Roizman, B. 1985. Isomerization of Herpes Simplex Virus 1 Genome: Identification of the Cis-Acting and Recombination Sites Within the Domain of the a Sequence. *Cell,* 41**,** 803-11.

Chou, J. & Roizman, B. 1986. The Terminal a Sequence of the Herpes Simplex Virus Genome Contains the Promoter of a Gene Located in the Repeat Sequences of the L Component. *J Virol,* 57**,** 629-37.

Choung, S., Kim, Y. J., Kim, S., Park, H. O. & Choi, Y. C. 2006. Chemical Modification of siRNAs to Improve Serum Stability Without Loss of Efficacy. *Biochem Biophys Res Commun,* 342**,** 919-27.

Christensen, M. H., Jensen, S. B., Miettinen, J. J., Luecke, S., Prabakaran, T., Reinert, L. S., Mettenleiter, T., Chen, Z. J., Knipe, D. M., Sandri-Goldin, R. M., Enquist, L. W., Hartmann, R., Mogensen, T. H., Rice, S. A., Nyman, T. A., Matikainen, S. & Paludan, S. R. 2016. HSV-1 ICP27 Targets the TBK1-Activated Sting Signalsome to Inhibit Virus-induced Type I IFN Expression. *Embo J,* 35**,** 1385-99.

Christophers, J., Clayton, J., Craske, J., Ward, R., Collins, P., Trowbridge, M. & Darby, G. 1998. Survey of Resistance of Herpes Simplex Virus to Acyclovir in Northwest England. *Antimicrob Agents Chemother,* 42**,** 868-72.

Clark, D. A., Freeland, M. L., Mackie, L. K., Jarrett, R. F. & Onions, D. E. 1993. Prevalence of Antibody to Human Herpesvirus 7 by Age. *J Infect Dis,* 168**,** 251-2.

Cohen, J. I. 2020. Herpesvirus Latency. *J Clin invest,* 130**,** 3361-3369.

Cokarić Brdovčak, M., Zubković, A., Ferenčić, A., Šoša, I., Stemberga, V., Cuculić, D., Rokić, F., Vugrek, O., Hackenberg, M. & Jurak, I. 2018. Herpes Simplex Virus 1 miRNA Sequence Variations in Latently Infected Human Trigeminal Ganglia. *Virus Res,* 256**,** 90-95.

Corey, L., Langenberg, A. G., ashley, R., Sekulovich, R. E., Izu, A. E., Douglas, J. M., Jr., Handsfield, H. H., Warren, T., Marr, L., Tyring, S., Dicarlo, R., Adimora, A. A., Leone, P., Dekker, C. L., Burke, R. L., Leong, W. P. & Straus, S. E. 1999. Recombinant Glycoprotein Vaccine for the Prevention of Genital HSV-2 Infection: Two Randomized Controlled Trials. Chiron HSV Vaccine Study Group. *Jama,* 282**,** 331-40.

Crameri, M., Bauer, M., Caduff, N., Walker, R., Steiner, F., Franzoso, F. D., Gujer, C., Boucke, K., Kucera, T., Zbinden, A., Münz, C., Fraefel, C., Greber, U. F. & Pavlovic, J. 2018. MxB is an Interferon-induced Restriction Factor of Human Herpesviruses. *Nat Commun,* 9**,** 1980.

Criscuolo, E., Castelli, M., Diotti, R. A., Amato, V., Burioni, R., Clementi, M., Ambrosi, A., Mancini, N. & Clementi, N. 2019. Cell-to-Cell Spread Blocking Activity is Extremely Limited in the Sera of Herpes Simplex Virus 1 (HSV-1)- and HSV-2-Infected Subjects. *J Virol,* 93.

Da Silva, A. S., Raposo, J. V., Pereira, T. C., Pinto, M. A. & De Paula, V. S. 2016. Effects of RNA Interference Therapy Against Herpes Simplex Virus Type 1 Encephalitis. *Antivir ther,* 21**,** 225-35.

Dahlgren, C., Zhang, H. Y., Du, Q., Grahn, M., Norstedt, G., Wahlestedt, C. & Liang, Z. 2008. Analysis of siRNA Specificity on Targets with Double-Nucleotide Mismatches. *Nucleic Acids Res,* 36**,** E53.

Dai, X. & Zhou, Z. H. 2018. Structure of the Herpes Simplex Virus 1 Capsid with Associated Tegument Protein Complexes. *Science,* 360.

Danve-Szatanek, C., Aymard, M., Thouvenot, D., Morfin, F., Agius, G., Bertin, I., Billaudel, S., Chanzy, B., Coste-Burel, M., Finkielsztejn, L., Fleury, H., Hadou, T., Henquell, C., Lafeuille, H., Lafon, M. E., Le Faou, A., Legrand, M. C., Maille, L., Mengelle, C., Morand, P., Morinet, F.,

Nicand, E., Omar, S., Picard, B., Pozzetto, B., Puel, J., Raoult, D., Scieux, C., Segondy, M., Seigneurin, J. M., Teyssou, R. & Zandotti, C. 2004. Surveillance Network for Herpes Simplex Virus Resistance to Antiviral Drugs: 3-Year Follow-Up. *J Clin Microbiol,* 42**,** 242-9.

Darville, J. M., Ley, B. E., Roome, A. P. & Foot, A. B. 1998. Acyclovir-Resistant Herpes Simplex Virus Infections in a Bone Marrow Transplant Population. *Bone Marrow Transplant,* 22**,** 587-9.

Dawson, C. R. & togni, B. 1976. Herpes Simplex Eye Infections: Clinical Manifestations, Pathogenesis and Management. *Surv Ophthalmol,* 21**,** 121-35.

De Bolle, L., Van Loon, J., De Clercq, E. & Naesens, L. 2005. Quantitative Analysis of Human Herpesvirus 6 Cell Tropism. *J Med Virol,* 75**,** 76-85.

Deiss, L. P., Chou, J. & Frenkel, N. 1986. Functional Domains Within the a Sequence Involved in the Cleavage-Packaging of Herpes Simplex Virus DNA. *J Virol,* 59**,** 605-18.

Devincenzo, J., Cehelsky, J. E., Alvarez, R., Elbashir, S., Harborth, J., toudjarska, I., Nechev, L., Murugaiah, V., Van Vliet, A., Vaishnaw, A. K. & Meyers, R. 2008. Evaluation of the Safety, Tolerability and Pharmacokinetics of Aln-Rsv01, a Novel RNAi Antiviral Therapeutic Directed Against Respiratory Syncytial Virus (Rsv). *Antiviral Res,* 77**,** 225-31.

Ding, S. W., Li, H., Lu, R., Li, F. & Li, W. X. 2004. RNA Silencing: a Conserved Antiviral Immunity of Plants and Animals. *Virus Res,* 102**,** 109-15.

Duan, R., De Vries, R. D., Osterhaus, A. D., Remeijer, L. & Verjans, G. M. 2008. Acyclovir-Resistant Corneal HSV-1 Isolates From Patients with Herpetic Keratitis. *J Infect Dis,* 198**,** 659-63.

Duan, R., De Vries, R. D., Van Dun, J. M., Van Loenen, F. B., Osterhaus, A. D., Remeijer, L. & Verjans, G. M. 2009. Acyclovir Susceptibility and Genetic Characteristics of Sequential Herpes Simplex Virus Type 1 Corneal Isolates From Patients with Recurrent Herpetic Keratitis. *J Infect Dis,* 200**,** 1402-14.

Dubin, G., Socolof, E., Frank, I. & Friedman, H. M. 1991. Herpes Simplex Virus Type 1 Fc Receptor Protects Infected Cells From Antibody-Dependent Cellular Cytotoxicity. *J Virol,* 65**,** 7046-50.

Edlefsen, P. T., Birkmann, A., Huang, M. L., Magaret, C. A., Kee, J. J., Diem, K., Goldner, T., Timmler, B., Stoelben, S., Ruebsamen-Schaeff, H., Zimmermann, H., Warren, T., Wald, A. & Corey, L. 2016. No Evidence of Pritelivir Resistance Among Herpes Simplex Virus Type 2 Isolates After 4 Weeks of Daily Therapy. *J Infect Dis,* 214**,** 258-64.

Egan, K., Hook, L. M., Latourette, P., Desmond, A., Awasthi, S. & Friedman, H. M. 2020a. Vaccines to Prevent Genital Herpes. *Transl Res,* 220**,** 138-152.

Egan, K. P., Hook, L. M., Naughton, A., Pardi, N., Awasthi, S., Cohen, G. H., Weissman, D. & Friedman, H. M. 2020b. an HSV-2 Nucleoside-Modified mRNA Genital Herpes Vaccine Containing Glycoproteins gC, gD, and gE Protects Mice Against HSV-1 Genital Lesions and Latent Infection. *Plos Pathog,* 16**,** E1008795.

Ejercito, P. M., Kieff, E. D. & Roizman, B. 1968. Characterization of Herpes Simplex Virus Strains Differing in their Effects on Social Behaviour of Infected Cells. *J Gen Virol,* 2**,** 357-64.

Fabozzi, G., Nabel, C. S., Dolan, M. A. & Sullivan, N. J. 2011. Ebolavirus Proteins Suppress the Effects of Small Interfering RNA by Direct Interaction with the Mammalian RNA Interference Pathway. *J Virol,* 85**,** 2512-23.

Farooq, A. V. & Shukla, D. 2012. Herpes Simplex Epithelial and Stromal Keratitis: an Epidemiologic Update. *Surv Ophthalmol,* 57**,** 448-62.

Fedorov, Y., anderson, E. M., Birmingham, A., Reynolds, A., Karpilow, J., Robinson, K., Leake, D., Marshall, W. S. & Khvorova, A. 2006. Off-Target Effects by siRNA Can Induce Toxic Phenotype. *RNA,* 12**,** 1188-96.

Fenwick, M. L. & Mcmenamin, M. M. 1984. Early Virion-associated Suppression of Cellular Protein Synthesis by Herpes Simplex Virus is Accompanied by Inactivation of mRNA. *J Gen Virol,* 65 (Pt 7)**,** 1225-8.

Foster, D. J., Brown, C. R., Shaikh, S., Trapp, C., Schlegel, M. K., Qian, K., Sehgal, A., Rajeev, K. G., Jadhav, V., Manoharan, M., Kuchimanchi, S., Maier, M. A. & Milstein, S. 2018. Advanced siRNA

Designs Further Improve In Vivo Performance of Galnac-siRNA Conjugates. *Mol ther,* 26**,** 708-717.

Frejborg, F., Kalke, K. & Hukkanen, V. 2022. Current Landscape in Antiviral Drug Development Against Herpes Simplex Virus Infections. *Smart Medicine*. e20220004

Friedman, H. M., Cohen, G. H., Eisenberg, R. J., Seidel, C. A. & Cines, D. B. 1984. Glycoprotein C of Herpes Simplex Virus 1 Acts as a Receptor for the C3b Complement Component on Infected Cells. *Nature,* 309**,** 633-5.

Früh, K., Ahn, K., Djaballah, H., Sempé, P., Van Endert, P. M., Tampé, R., Peterson, P. A. & Yang, Y. 1995. A Viral Inhibitor of Peptide Transporters for Antigen Presentation. *Nature,* 375**,** 415-8.

Fucini, R. V., Haringsma, H. J., Deng, P., Flanagan, W. M. & Willingham, A. T. 2012. Adenosine Modification May Be Preferred for Reducing siRNA Immune Stimulation. *Nucleic Acid ther,* 22**,** 205-10.

Garber, D. A., Beverley, S. M. & Coen, D. M. 1993. Demonstration of Circularization of Herpes Simplex Virus DNA Following Infection Using Pulsed Field Gel Electrophoresis. *Virology,* 197**,** 459-62.

Grünewald, K., Desai, P., Winkler, D. C., Heymann, J. B., Belnap, D. M., Baumeister, W. & Steven, A. C. 2003. Three-Dimensional Structure of Herpes Simplex Virus from Cryo-Electron Tomography. *Science,* 302**,** 1396-8.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. 1975. Anatomy of Herpes Simplex Virus DNA: Evidence for Four Populations of Molecules that Differ in the Relative Orientations of their Long and Short Components. *Proc Natl Acad Sci U S A,* 72**,** 4243-7.

He, B., Gross, M. & Roizman, B. 1997. the Gamma(1)34.5 Protein of Herpes Simplex Virus 1 Complexes with Protein Phosphatase 1alpha to Dephosphorylate the Alpha Subunit of the Eukaryotic Translation Initiation Factor 2 and Preclude the Shutoff of Protein Synthesis by Double-Stranded RNA-Activated Protein Kinase. *Proc Natl Acad Sci U S A,* 94**,** 843-8.

Herpescureadvocacy.com. 2022. Herpes Cure Pipeline 2.0. Accessed 03.01.2022. Available at: https://herpescureadvocacy.com/2022/03/17/herpes-cure-pipeline-2-0-2/

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. & Johnson, D. 1995. Herpes Simplex Virus Turns Off the Tap to Evade Host Immunity. *Nature,* 375**,** 411-5.

Hill, J. M., Quenelle, D. C., Cardin, R. D., Vogel, J. L., Clement, C., Bravo, F. J., Foster, T. P., Bosch-Marce, M., Raja, P., Lee, J. S., Bernstein, D. I., Krause, P. R., Knipe, D. M. & Kristie, T. M. 2014. Inhibition of LSD1 Reduces Herpesvirus Infection, Shedding, and Recurrence by Promoting Epigenetic Suppression of Viral Genomes. *Sci Transl Med,* 6**,** 265ra169.

Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E. & Prydz, H. 2002. Positional Effects of Short Interfering RNAs Targeting the Human Coagulation Trigger Tissue Factor. *Nucleic Acids Res,* 30**,** 1757-66.

Honess, R. W. & Roizman, B. 1974. Regulation of Herpesvirus Macromolecular Synthesis. I. Cascade Regulation of the Synthesis of Three Groups of Viral Proteins. *J Virol,* 14**,** 8-19.

Isaacs, A. & Lindenmann, J. 1957. Virus Interference. I. The Interferon. *Proc R Soc Lond B Biol Sci,* 147**,** 258-67.

Jackson, A. L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J. M., Lim, L., Karpilow, J., Nichols, K., Marshall, W., Khvorova, A. & Linsley, P. S. 2006. Position-Specific Chemical Modification of siRNAs Reduces "Off-Target" Transcript Silencing. *RNA,* 12**,** 1197-205.

James, S. & Kimberlin, D. 2015. Neonatal Herpes Simplex Virus Infection : Epidemiology and Treatment. 42(1)**,** 47-59.

Janas, M. M., Zlatev, I., Liu, J., Jiang, Y., Barros, S. A., Sutherland, J. E., Davis, W. P., Brown, C. R., Liu, X., Schlegel, M. K., Blair, L., Zhang, X., Das, B., Tran, C., Aluri, K., Li, J., Agarwal, S., indrakanti, R., Charisse, K., Nair, J., Matsuda, S., Rajeev, K. G., Zimmermann, T., Sepp-Lorenzino, L., Xu, Y., Akinc, A., Fitzgerald, K., Vaishnaw, A. K., Smith, P. F., Manoharan, M.,

Jadhav, V., Wu, J. T. & Maier, M. A. 2019. Safety Evaluation of 2'-Deoxy-2'-Fluoro Nucleotides in GalNac-siRNA Conjugates. *Nucleic Acids Res,* 47**,** 3306-3320.

Jiang, M., Osterlund, P., Sarin, L. P., Poranen, M. M., Bamford, D. H., Guo, D. & Julkunen, I. 2011. Innate Immune Responses in Human Monocyte-Derived Dendritic Cells Are Highly Dependent on the Size and the 5' Phosphorylation of RNA Molecules. *J Immunol,* 187**,** 1713-21.

Jiang, M., Österlund, P., Westenius, V., Guo, D., Poranen, M. M., Bamford, D. H. & Julkunen, I. 2019. Efficient Inhibition of Avian and Seasonal Influenza A Viruses by a Virus-Specific Dicer-Substrate Small Interfering RNA Swarm in Human Monocyte-Derived Macrophages and Dendritic Cells. *J Virol,* 93.

Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M. & Stow, N. D. 1988. Herpes Simplex Virus Immunoglobulin G Fc Receptor Activity Depends on a Complex of Two Viral Glycoproteins, gE and gI. *J Virol,* 62**,** 1347-54.

Kalamvoki, M. & Roizman, B. 2010. Circadian Clock Histone Acetyl Transferase Localizes at ND10 Nuclear Bodies and Enables Herpes Simplex Virus Gene Expression. *Proc Natl Acad Sci U S A,* 107**,** 17721-6.

Kalke, K. 2020. *Treatment of Herpes Simplex Virus Infection Using Antiviral siRNA Swarms with 2'-fluoro-Modifications.* Master's thesis, Institute of Biomedicine, University of Turku, Turku, Finland.

Kalke, K., Orpana, J., Lasanen, T., Esparta, O., Lund, L. M., Frejborg, F., Vuorinen, T., Paavilainen, H. & Hukkanen, V. 2022. The In Vitro Replication, Spread, and Oncolytic Potential of Finnish Circulating Strains of Herpes Simplex Virus Type 1. *Viruses,* 14**,** 1290.

Kenski, D. M., Butora, G., Willingham, A. T., Cooper, A. J., Fu, W., Qi, N., Soriano, F., Davies, I. W. & Flanagan, W. M. 2012. siRNA-Optimized Modifications for Enhanced In Vivo Activity. *Mol ther Nucleic Acids,* 1**,** E5.

Kim, D. H., Behlke, M. A., Rose, S. D., Chang, M. S., Choi, S. & Rossi, J. J. 2005. Synthetic DsRNA Dicer Substrates Enhance RNAi Potency and Efficacy. *Nat Biotechnol,* 23**,** 222-6.

Kimberlin, D. W., Coen, D. M., Biron, K. K., Cohen, J. I., Lamb, R. A., Mckinlay, M., Emini, E. A. & Whitley, R. J. 1995. Molecular Mechanisms of Antiviral Resistance. *Antiviral Res,* 26**,** 369-401.

Kortekangas-Savolainen, O., Orhanen, E., Puodinketo, T. & Vuorinen, T. 2014. Epidemiology of Genital Herpes Simplex Virus Type 1 and 2 Infections in Southwestern Finland During a 10-Year Period (2003-2012). *Sex Transm Dis,* 41**,** 268-71.

Kose, S., Mandiracioglu, A., Senger, S. S., ULu, Y., Cavdar, G., Gol, B., Gurbuz, I., Sariavci, S. & Nohutcu, N. 2013. Seroprevalence of Varicella-Zoster Virus in the Prevaccine Era: A Population-Based Study in Izmir, Turkey. *J Infect Public Health,* 6**,** 115-9.

Koulu, M. & Mervaala, E. 2013. *Farmakologia ja toksikologia,* Kuopio, Finland, Kustannusosakeyhtiö Medicina.

Kristie, T. M. & Roizman, B. 1987. Host Cell Proteins Bind to the Cis-Acting Site Required for Virion-Mediated Induction of Herpes Simplex Virus 1 Alpha Genes. *Proc Natl Acad Sci U S A,* 84**,** 71-5.

Ku, C. C., Che, X. B., Reichelt, M., Rajamani, J., Schaap-Nutt, A., Huang, K. J., Sommer, M. H., Chen, Y. S., Chen, Y. Y. & Arvin, A. M. 2011. Herpes Simplex Virus-1 Induces Expression of a Novel MxA Isoform That Enhances Viral Replication. *Immunol Cell Biol,* 89**,** 173-82.

Kurt-Jones, E. A., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., Arnold, M. M., Knipe, D. M. & Finberg, R. W. 2004. Herpes Simplex Virus 1 Interaction with Toll-Like Receptor 2 Contributes to Lethal Encephalitis. *Proc Natl Acad Sci U S A,* 101**,** 1315-20.

Kwong, A. D. & Frenkel, N. 1989. The Herpes Simplex Virus Virion Host Shutoff Function. *J Virol,* 63**,** 4834-9.

Kwong, A. D., Kruper, J. A. & Frenkel, N. 1988. Herpes Simplex Virus Virion Host Shutoff Function. *J Virol,* 62**,** 912-21.

Labetoulle, M., Auquier, P., Conrad, H., Crochard, A., Daniloski, M., Bouee, S., El Hasnaoui, A. & Colin, J. 2005. Incidence of Herpes Simplex Virus Keratitis in France. *Ophthalmology,* 112**,** 888-95.

Labetoulle, M., Kucera, P., Ugolini, G., Lafay, F., Frau, E., Offret, H. & Flamand, A. 2000. Neuronal Propagation of HSV1 From the Oral Mucosa to the Eye. *Invest Ophthalmol Vis Sci,* 41**,** 2600-6.

Labetoulle, M., Maillet, S., Efstathiou, S., Dezelee, S., Frau, E. & Lafay, F. 2003. HSV1 Latency Sites After Inoculation in the Lip: Assessment of Their Localization and Connections to the Eye. *Invest Ophthalmol Vis Sci,* 44**,** 217-25.

Lam, J. K., Chow, M. Y., Zhang, Y. & Leung, S. W. 2015. siRNA Versus miRNA as Therapeutics for Gene Silencing. *Mol ther Nucleic Acids,* 4**,** E252.

Leino, T. & Puumalainen, T. 2018. Vesirokkorokotus Rokotusohjelmassa. *Duodecim,* 134**,** 1770-2.

Levanova, A. & Poranen, M. M. 2018. RNA Interference as a Prospective Tool for the Control of Human Viral Infections. *Front Microbiol,* 9**,** 2151.

Li, Y., Basavappa, M., Lu, J., Dong, S., Cronkite, D. A., Prior, J. T., Reinecker, H. C., Hertzog, P., Han, Y., Li, W. X., Cheloufi, S., Karginov, F. V., Ding, S. W. & Jeffrey, K. L. 2016. Induction and Suppression of Antiviral RNA Interference by Influenza a Virus in Mammalian Cells. *Nat Microbiol,* 2**,** 16250.

Li, Y., Song, Y., Zhu, L., Wang, X., Richers, B., Leung, D. Y. M. & Bin, L. 2020. Interferon Kappa is Important for Keratinocyte Host Defense Against Herpes Simplex Virus-1. *J Immunol Res,* 2020**,** 5084682.

Li, Z., Duan, F., Lin, L., Huang, Q. & Wu, K. 2014. A New Approach of Delivering siRNA to the Cornea and Its Application for Inhibiting Herpes Simplex Keratitis. *Curr Mol Med,* 14**,** 1215-25.

Liang, Y., Quenelle, D., Vogel, J. L., Mascaro, C., Ortega, A. & Kristie, T. M. 2013. A Novel Selective Lsd1/Kdm1a Inhibitor Epigenetically Blocks Herpes Simplex Virus Lytic Replication and Reactivation From Latency. *Mbio,* 4**,** E00558-12.

Linderman, J. A., Kobayashi, M., Rayannavar, V., Fak, J. J., Darnell, R. B., Chao, M. V., Wilson, A. C. & Mohr, I. 2017. Immune Escape via a Transient Gene Expression Program Enables Productive Replication of a Latent Pathogen. *Cell Rep,* 18**,** 1312-1323.

Lizarraga, K. J., Alexandre, L. C., Ramos-Estebanez, C. & Merenda, A. 2013. Are Steroids a Beneficial Adjunctive Therapy in the Immunosuppressed Patient with Herpes Simplex Virus Encephalitis? *Case Rep Neurol,* 5**,** 52-5.

Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M., Vickerman, P., Gottlieb, S. L. & Newman, L. M. 2015a. Global and Regional Estimates of Prevalent and Incident Herpes Simplex Virus Type 1 Infections in 2012. *Plos One,* 10**,** E0140765.

Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M. E., Vickerman, P., Newman, L. M. & Gottlieb, S. L. 2017. First Estimates of the Global and Regional Incidence of Neonatal Herpes Infection. *Lancet Glob Health,* 5**,** E300-E309.

Looker, K. J., Magaret, A. S., Turner, K. M., Vickerman, P., Gottlieb, S. L. & Newman, L. M. 2015b. Global Estimates of Prevalent and Incident Herpes Simplex Virus Type 2 Infections in 2012. *Plos One,* 10**,** E114989.

Loret, S., Guay, G. & Lippé, R. 2008. Comprehensive Characterization of Extracellular Herpes Simplex Virus Type 1 Virions. *J Virol,* 82**,** 8605-18.

Macrae, I. J., Li, F., Zhou, K., Cande, W. Z. & DouDNA, J. A. 2006. Structure of Dicer and Mechanistic Implications for RNAi. *Cold Spring Harb Symp Quant Biol,* 71**,** 73-80.

Manoharan, M., Akinc, A., Pandey, R. K., Qin, J., Hadwiger, P., John, M., Mills, K., Charisse, K., Maier, M. A., Nechev, L., Greene, E. M., Pallan, P. S., Rozners, E., Rajeev, K. G. & Egli, M. 2011. Unique Gene-Silencing and Structural Properties of 2'-fluoro-modified siRNAs. *Angew Chem int Ed Engl,* 50**,** 2284-8.

Mattila, R. K., Harila, K., Kangas, S. M., Paavilainen, H., Heape, A. M., Mohr, I. J. & Hukkanen, V. 2015. An Investigation of Herpes Simplex Virus Type 1 Latency in a Novel Mouse Dorsal Root Ganglion Model Suggests a Role for ICP34.5 in Reactivation. *J Gen Virol,* 96**,** 2304-2313.

Mcgeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., Mcnab, D., Perry, L. J., Scott, J. E. & Taylor, P. 1988a. The Complete DNA Sequence of the Long Unique Region in the Genome of Herpes Simplex Virus Type 1. *J Gen Virol,* 69 (Pt 7)**,** 1531-74.

Mcgeoch, D. J., Dalrymple, M. A., Dolan, A., Mcnab, D., Perry, L. J., Taylor, P. & Challberg, M. D. 1988b. Structures of Herpes Simplex Virus Type 1 Genes Required for Replication of Virus DNA. *J Virol,* 62**,** 444-53.

Mcgeoch, D. J., Dolan, A., Donald, S. & Brauer, D. H. 1986. Complete DNA Sequence of the Short Repeat Region in the Genome of Herpes Simplex Virus Type 1. *Nucleic Acids Res,* 14**,** 1727-45.

Mcknight, J. L., Kristie, T. M. & Roizman, B. 1987. Binding of the Virion Protein Mediating Alpha Gene Induction in Herpes Simplex Virus 1-Infected Cells to its Cis Site Requires Cellular Proteins. *Proc Natl Acad Sci U S A,* 84**,** 7061-5.

Mclaren, C., Sibrack, C. D. & Barry, D. W. 1982. Spectrum of Sensitivity of Acyclovir of Herpes Simplex Virus Clinical Isolates. *Am J Med,* 73**,** 376-9.

Mctavish, H., Zerebiec, K. W., Zeller, J. C., Shekels, L. L., Matson, M. A. & Kren, B. T. 2019. Immune Characteristics Correlating with HSV-1 Immune Control and Effect of Squaric Acid Dibutyl Ester on Immune Characteristics of Subjects with Frequent Herpes Labialis Episodes. *Immun inflamm Dis,* 7**,** 22-40.

Minkovitz, J. B. & Pepose, J. S. 1995. Topical Interferon Alpha-2a Treatment of Herpes Simplex Keratitis Resistant to Multiple Antiviral Medications in an Immunosuppressed Patient. *Cornea,* 14**,** 326-30.

Mocarski, E. S. & Roizman, B. 1981. Site-Specific Inversion Sequence of the Herpes Simplex Virus Genome: Domain and Structural Features. *Proc Natl Acad Sci U S A,* 78**,** 7047-51.

Mocarski, E. S. & Roizman, B. 1982. Structure and Role of the Herpes Simplex Virus DNA Termini in Inversion, Circularization and Generation of Virion DNA. *Cell,* 31**,** 89-97.

Moon, J. S., Lee, S. H., Kim, E. J., Cho, H., Lee, W., Kim, G. W., Park, H. J., Cho, S. W., Lee, C. & Oh, J. W. 2016. Inhibition of Hepatitis C Virus in Mice by a Small Interfering RNA Targeting a Highly Conserved Sequence in Viral IRES Pseudoknot. *Plos One,* 11**,** E0146710.

Morfin, F., Bilger, K., Boucher, A., Thiebaut, A., Najioullah, F., Bleyzac, N., Raus, N., Bosshard, S., Aymard, M., Michallet, M. & Thouvenot, D. 2004. HSV Excretion After Bone Marrow Transplantation: A 4-Year Survey. *J Clin Virol,* 30**,** 341-5.

Muhonen, P., Tennila, T., Azhayeva, E., Parthasarathy, R. N., Janckila, A. J., Vaananen, H. K., Azhayev, A. & Laitala-Leinonen, T. 2007. RNA Interference Tolerates 2'-Fluoro Modifications at the Argonaute2 Cleavage Site. *Chem Biodivers,* 4**,** 858-73.

Mäki, J., Paavilainen, H., Grenman, S., Syrjanen, S. & Hukkanen, V. 2015. Carriage of Herpes Simplex Virus and Human Papillomavirus in Oral Mucosa is Rare in Young Women: A Long-Term Prospective Follow-Up. *J Clin Virol,* 70**,** 58-62.

Mäki, J., Paavilainen, H., Kero, K., Hukkanen, V. & Syrjanen, S. 2018. Herpes Simplex and Human Papilloma Virus Coinfections in Oral Mucosa of Men-A 6-Year Follow-Up Study. *J Med Virol,* 90**,** 564-570.

Neumann, J., Eis-Hübinger, A. M. & Koch, N. 2003. Herpes Simplex Virus Type 1 Targets the MHC Class II Processing Pathway for Immune Evasion. *J Immunol,* 171**,** 3075-83.

Nguyen, D. N., Chen, S. C., Lu, J., Goldberg, M., Kim, P., Sprague, A., Novobrantseva, T., Sherman, J., Shulga-Morskaya, S., De Fougerolles, A., Chen, J., Langer, R. & Anderson, D. G. 2009. Drug Delivery-Mediated Control of RNA Immunostimulation. *Mol ther,* 17**,** 1555-62.

Nicola, A. V., Hou, J., Major, E. O. & Straus, S. E. 2005. Herpes Simplex Virus Type 1 Enters Human Epidermal Keratinocytes, but not Neurons, via a Ph-Dependent Endocytic Pathway. *J Virol,* 79**,** 7609-16.

Nicoll, M. P., Proenca, J. T. & Efstathiou, S. 2012. The Molecular Basis of Herpes Simplex Virus Latency. *Fems Microbiol Rev,* 36**,** 684-705.

Nygårdas, M., Vuorinen, T., Aalto, A. P., Bamford, D. H. & Hukkanen, V. 2009. Inhibition of Coxsackievirus B3 and Related Enteroviruses by Antiviral Short Interfering RNA Pools Produced Using Phi6 RNA-Dependent RNA Polymerase. *J Gen Virol,* 90**,** 2468-73.

Orvedahl, A., Alexander, D., Tallóczy, Z., Sun, Q., Wei, Y., Zhang, W., Burns, D., Leib, D. A. & Levine, B. 2007. HSV-1 ICP34.5 Confers Neurovirulence by Targeting the Beclin 1 Autophagy Protein. *Cell Host Microbe,* 1**,** 23-35.

Orzalli, M. H., Deluca, N. A. & Knipe, D. M. 2012. Nuclear IFI16 induction of IRF-3 Signaling During Herpesviral Infection and Degradation of IFI16 by the Viral ICP0 Protein. *Proc Natl Acad Sci U S A,* 109**,** E3008-17.

Owen, D. J., Crump, C. M. & Graham, S. C. 2015. Tegument Assembly and Secondary Envelopment of Alphaherpesviruses. *Viruses,* 7**,** 5084-114.

Paavilainen, H. 2017. *Inhibiton of Herpes Simplex Virus Infection with RNA Interference*, University of Turku, Finland.

Paavilainen, H., Lehtinen, J., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. 2016. Inhibition of Clinical Pathogenic Herpes Simplex Virus 1 Strains with Enzymatically Created siRNA Pools. *J Med Virol,* 88**,** 2196-2205.

Paavilainen, H., Lehtinen, J., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. 2017. Topical Treatment of Herpes Simplex Virus Infection with Enzymatically Created siRNA Swarm. *Antivir ther,* 22**,** 631-637.

Paavilainen, H., Romanovskaya, A., Nygårdas, M., Bamford, D. H., Poranen, M. M. & Hukkanen, V. 2015. Innate Responses to Small Interfering RNA Pools Inhibiting Herpes Simplex Virus Infection in Astrocytoid and Epithelial Cells. *Innate Immun,* 21**,** 349-57.

Palliser, D., Chowdhury, D., Wang, Q. Y., Lee, S. J., Bronson, R. T., Knipe, D. M. & Lieberman, J. 2006. An siRNA-Based Microbicide Protects Mice from Lethal Herpes Simplex Virus 2 Infection. *Nature,* 439**,** 89-94.

Pan, D., Kaye, S. B., Hopkins, M., Kirwan, R., Hart, I. J. & Coen, D. M. 2014. Common and New Acyclovir Resistant Herpes Simplex Virus-1 Mutants Causing Bilateral Recurrent Herpetic Keratitis in an Immunocompetent Patient. *J Infect Dis,* 209**,** 345-9.

Parris, D. S. & Harrington, J. E. 1982. Herpes Simplex Virus Variants Restraint to High Concentrations of Acyclovir Exist in Clinical Isolates. *Antimicrob Agents Chemother,* 22**,** 71-7.

Parsons, B. D., Schindler, A., Evans, D. H. & Foley, E. 2009. A Direct Phenotypic Comparison of siRNA Pools and Multiple individual Duplexes in a Functional assay. *Plos One,* 4**,** E8471.

Parsons, L. R., Tafuri, Y. R., Shreve, J. T., Bowen, C. D., Shipley, M. M., Enquist, L. W. & Szpara, M. L. 2015. Rapid Genome Assembly and Comparison Decode Intrastrain Variation in Human Alphaherpesviruses. *Mbio,* 6.

Pellett, P. E., Mcknight, J. L., Jenkins, F. J. & Roizman, B. 1985. Nucleotide Sequence and Predicted Amino Acid Sequence of a Protein Encoded in a Small Herpes Simplex Virus DNA Fragment Capable of Trans-Inducing Alpha Genes. *Proc Natl Acad Sci U S A,* 82**,** 5870-4.

Peri, P., Mattila, R. K., Kantola, H., Broberg, E., Karttunen, H. S., Waris, M., Vuorinen, T. & Hukkanen, V. 2008. Herpes Simplex Virus Type 1 US3 Gene Deletion Influences Toll-Like Receptor Responses in Cultured Monocytic Cells. *Virol J,* 5**,** 140.

Perry, C. M. & Balfour, J. A. 1999. Fomivirsen. *Drugs,* 57**,** 375-80; Discussion 381.

Post, L. E., Mackem, S. & Roizman, B. 1981. Regulation of Alpha Genes of Herpes Simplex Virus: Expression of Chimeric Genes Produced by Fusion of Thymidine Kinase with Alpha Gene Promoters. *Cell,* 24**,** 555-65.

Prakash, T. P., Allerson, C. R., Dande, P., Vickers, T. A., Sioufi, N., Jarres, R., Baker, B. F., Swayze, E. E., Griffey, R. H. & Bhat, B. 2005. Positional Effect of Chemical Modifications on Short Interference RNA Activity in Mammalian Cells. *J Med Chem,* 48**,** 4247-53.

Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B. & Radmark, O. 2002. Ribonuclease Activity and RNA Binding of Recombinant Human Dicer. *Embo J,* 21**,** 5864-74.

Puhakka, L., Sarvikivi, E., Lappalainen, M., Surcel, H. M. & Saxen, H. 2016. Decrease in Seroprevalence for Herpesviruses Among Pregnant Women in Finland: Cross-Sectional Study of Three Time Points 1992, 2002 and 2012. *Infect Dis (Lond),* 48**,** 406-10.

Qiu, Y., Xu, Y., Zhang, Y., Zhou, H., Deng, Y. Q., Li, X. F., Miao, M., Zhang, Q., Zhong, B., Hu, Y., Zhang, F. C., Wu, L., Qin, C. F. & Zhou, X. 2017. Human Virus-Derived Small RNAs Can Confer Antiviral Immunity in Mammals. *Immunity,* 46**,** 992-1004 E5.

Ramos-Estebanez, C., Lizarraga, K. J. & Merenda, A. 2014. A Systematic Review on the Role of Adjunctive Corticosteroids in Herpes Simplex Virus Encephalitis: Is Timing Critical for Safety and Efficacy? *Antivir ther,* 19**,** 133-9.

Ramsey, N. L. M., Visciano, M., Hunte, R., Loh, L. N., Burn Aschner, C., Jacobs, W. R., Jr. & Herold, B. C. 2020. A Single-Cycle Glycoprotein D Deletion Viral Vaccine Candidate, DeltagD-2, Elicits Polyfunctional Antibodies That Protect Against Ocular Herpes Simplex Virus. *J Virol,* 94.

Rand, T. A., Petersen, S., Du, F. & Wang, X. 2005. Argonaute2 Cleaves the Anti-Guide Strand of siRNA During RISC Activation. *Cell,* 123**,** 621-9.

Ranta, V. P., Laavola, M., Toropainen, E., Vellonen, K. S., Talvitie, A. & Urtti, A. 2003. Ocular Pharmacokinetic Modeling Using Corneal Absorption and Desorption Rates From In Vitro Permeation Experiments with Cultured Corneal Epithelial Cells. *Pharm Res,* 20**,** 1409-16.

Raschilas, F., Wolff, M., Delatour, F., Chaffaut, C., De Broucker, T., Chevret, S., Lebon, P., Canton, P. & Rozenberg, F. 2002. Outcome of and Prognostic Factors for Herpes Simplex Encephalitis in Adult Patients: Results of a Multicenter Study. *Clin Infect Dis,* 35**,** 254-60.

Read, G. S. & Frenkel, N. 1983. Herpes Simplex Virus Mutants Defective in the Virion-Associated Shutoff of Host Polypeptide Synthesis and Exhibiting Abnormal Synthesis of Alpha (Immediate Early) Viral Polypeptides. *J Virol,* 46**,** 498-512.

Reynolds, A., anderson, E. M., Vermeulen, A., Fedorov, Y., Robinson, K., Leake, D., Karpilow, J., Marshall, W. S. & Khvorova, A. 2006. Induction of the Interferon Response by siRNA is Cell Type- and Duplex Length-Dependent. *RNA,* 12**,** 988-93.

Rodriguez, M., Lapierre, J., Ojha, C. R., Kaushik, A., Batrakova, E., Kashanchi, F., Dever, S. M., Nair, M. & El-Hage, N. 2018. Intranasal Drug Delivery of Small Interfering RNA Targeting Beclin1 Encapsulated with Polyethylenimine (Pei) in Mouse Brain to Achieve HIV Attenuation. *Sci Rep,* 8**,** 4778.

Rohner, E., Wyss, N., Trelle, S., Mbulaiteye, S. M., Egger, M., Novak, U., Zwahlen, M. & Bohlius, J. 2014. HHV-8 Seroprevalence: a Global View. *Syst Rev,* 3**,** 11.

Romanovskaya, A., Paavilainen, H., Nygårdas, M., Bamford, D. H., Hukkanen, V. & Poranen, M. M. 2012. Enzymatically Produced Pools of Canonical and Dicer-Substrate siRNA Molecules Display Comparable Gene Silencing and Antiviral Activities Against Herpes Simplex Virus. *Plos One,* 7**,** E51019.

Romanovskaya, A., Sarin, L. P., Bamford, D. H. & Poranen, M. M. 2013. High-Throughput Purification of Double-Stranded RNA Molecules Using Convective Interaction Media Monolithic Anion Exchange Columns. *J Chromatogr A,* 1278**,** 54-60.

Rönkkö, S., Vellonen, K. S., Järvinen, K., Toropainen, E. & Urtti, A. 2016. Human Corneal Cell Culture Models for Drug Toxicity Studies. *Drug Deliv Transl Res,* 6**,** 660-675.

Sadegh Ehdaei, B., Pirouzmand, A., Shabani, M., Mirzaei, A. & Moghim, S. 2021. Cellular Mir-101-1 Reduces Efficiently the Replication of HSV-1 in HeLa Cells. *Intervirology,* 64**,** 88-95.

Sanbaumhüter, M., Döhner, K., Schipke, J., Binz, A., Pohlmann, A., Sodeik, B. & Bauerfeind, R. 2012. Cytosolic Herpes Simplex Virus Capsids not only Require Binding Inner Tegument Protein Pul36 but also Pul37 for Active Transport Prior to Secondary Envelopment. *Cellular Microbiology,* 15**,** 248-269.

Sato, Y., Suenaga, T., Kobayashi, M., Miyazaki, N., Suzuki, T., Ishioka, K. & Suzutani, T. 2021. Characteristics of Helicase-Primase Inhibitor Amenamevir-Resistant Herpes Simplex Virus. *Antimicrob Agents Chemother,* 65**,** E0049421.

Sauerbrei, A., Bohn, K., Heim, A., Hofmann, J., Weissbrich, B., Schnitzler, P., Hoffmann, D., Zell, R., Jahn, G., Wutzler, P. & Hamprecht, K. 2011. Novel Resistance-Associated Mutations of Thymidine Kinase and DNA Polymerase Genes of Herpes Simplex Virus Type 1 and Type 2. *Antivir ther,* 16**,** 1297-308.

Sayers, C. L. & Elliott, G. 2016. Herpes Simplex Virus 1 Enters Human Keratinocytes by a Nectin-1-Dependent, Rapid Plasma Membrane Fusion Pathway That Functions at Low Temperature. *J Virol,* 90**,** 10379-10389.

Schlee, M., Hornung, V. & Hartmann, G. 2006. siRNA and isRNA: Two Edges of One Sword. *Mol ther,* 14**,** 463-70.

Schmidt, S., Bohn-Wippert, K., Schlattmann, P., Zell, R. & Sauerbrei, A. 2015. Sequence Analysis of Herpes Simplex Virus 1 Thymidine Kinase and DNA Polymerase Genes From Over 300 Clinical Isolates From 1973 to 2014 Finds Novel Mutations That May Be Relevant for Development of Antiviral Resistance. *Antimicrob Agents Chemother,* 59**,** 4938-45.

Schnipper, L. E., Crumpacker, C. S., Marlowe, S. I., Kowalsky, P., Hershey, B. J. & Levin, M. J. 1982. Drug-Resistant Herpes Simplex Virus In Vitro and After Acyclovir Treatment in an Immunocompromised Patient. *Am J Med,* 73**,** 387-92.

Schubert, S., Rothe, D., Werk, D., Grunert, H. P., Zeichhardt, H., Erdmann, V. A. & Kurreck, J. 2007. Strand-Specific Silencing of a Picornavirus by RNA Interference: Evidence for the Superiority of Plus-Strand Specific siRNAs. *Antiviral Res,* 73**,** 197-205.

Shipley, M. M., Renner, D. W., Ott, M., Bloom, D. C., Koelle, D. M., Johnston, C. & Szpara, M. L. 2018. Genome-Wide Surveillance of Genital Herpes Simplex Virus Type 1 From Multiple Anatomic Sites Over Time. *J Infect Dis,* 218**,** 595-605.

Sibrack, C. D., Gutman, L. T., Wilfert, C. M., Mclaren, C., St Clair, M. H., Keller, P. M. & Barry, D. W. 1982. Pathogenicity of Acyclovir-Resistant Herpes Simplex Virus Type 1 From an Immunodeficient Child. *J Infect Dis,* 146**,** 673-82.

Smith, K. O. 1964. Relationship Between the Envelope and the Infectivity of Herpes Simplex Virus. *Proc Soc Exp Biol Med,* 115**,** 814-6.

Snijder, B., Sacher, R., Rämö, P., Liberali, P., Mench, K., Wolfrum, N., Burleigh, L., Scott, C. C., Verheije, M. H., Mercer, J., Moese, S., Heger, T., theusner, K., Jurgeit, A., Lamparter, D., Balistreri, G., Schelhaas, M., De Haan, C. A., Marjomäki, V., Hyypiä, T., Rottier, P. J., Sodeik, B., Marsh, M., Gruenberg, J., Amara, A., Greber, U., Helenius, A. & Pelkmans, L. 2012. Single-Cell Analysis of Population Context Advances RNAi Screening at Multiple Levels. *Mol Syst Biol,* 8**,** 579.

Sodeik, B., Ebersold, M. W. & Helenius, A. 1997. Microtubule-Mediated Transport of Incoming Herpes Simplex Virus 1 Capsids to the Nucleus. *J Cell Biol,* 136**,** 1007-21.

Stanberry, L. R., Spruance, S. L., Cunningham, A. L., Bernstein, D. I., Mindel, A., Sacks, S., Tyring, S., Aoki, F. Y., Slaoui, M., Denis, M., Vandepapeliere, P., Dubin, G. & GlaxoSmithKline Herpes Vaccine Efficacy Study, G. 2002. Glycoprotein-D-Adjuvant Vaccine to Prevent Genital Herpes. *N Engl J Med,* 347**,** 1652-61.

Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L. & Feldman, L. T. 1987. RNA Complementary to a Herpesvirus Alpha Gene mRNA is Prominent in Latently Infected Neurons. *Science,* 235**,** 1056-9.

Strang, B. L. & Stow, N. D. 2005. Circularization of the Herpes Simplex Virus Type 1 Genome Upon Lytic Infection. *J Virol,* 79**,** 12487-94.

Stranska, R., Schuurman, R., Nienhuis, E., Goedegebuure, I. W., Polman, M., Weel, J. F., Wertheim-Van Dillen, P. M., Berkhout, R. J. & Van Loon, A. M. 2005. Survey of Acyclovir-Resistant Herpes Simplex Virus in the Netherlands: Prevalence and Characterization. *J Clin Virol,* 32**,** 7-18.

Tarn, W. Y., Cheng, Y., Ko, S. H. & Huang, L. M. 2021. Antisense Oligonucleotide-Based Therapy of Viral Infections. *Pharmaceutics,* 13.

Tatiparti, K., Sau, S., Kashaw, S. K. & Iyer, A. K. 2017. siRNA Delivery Strategies: A Comprehensive Review of Recent Developments. *Nanomaterials (Basel),* 7.

Toropainen, E., Ranta, V. P., Talvitie, A., Suhonen, P. & Urtti, A. 2001. Culture Model of Human Corneal Epithelium for Prediction of Ocular Drug Absorption. *invest Ophthalmol Vis Sci,* 42**,** 2942-8.

Toropainen, E., Ranta, V. P., Vellonen, K. S., Palmgrén, J., Talvitie, A., Laavola, M., Suhonen, P., Hämäläinen, K. M., Auriola, S. & Urtti, A. 2003. Paracellular and Passive Transcellular Permeability in Immortalized Human Corneal Epithelial Cell Culture Model. *Eur J Pharm Sci,* 20**,** 99-106.

Tuokko, H., Bloigu, R. & Hukkanen, V. 2014. Herpes Simplex Virus Type 1 Genital Herpes in Young Women: Current Trend in Northern Finland. *Sex Transm Infect,* 90**,** 160.

Tzellos, S. & Farrell, P. J. 2012. Epstein-Barr Virus Sequence Variation-Biology and Disease. *Pathogens,* 1**,** 156-74.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. 2008. MicroRNAs Expressed by Herpes Simplex Virus 1 During Latent Infection Regulate Viral mRNAs. *Nature,* 454**,** 780-3.

Wald, A., Timmler, B., Magaret, A., Warren, T., Tyring, S., Johnston, C., Fife, K., Selke, S., Huang, M. L., StobeRNAck, H. P., Zimmermann, H., Corey, L., Birkmann, A. & Ruebsamen-Schaeff, H. 2016. Effect of Pritelivir Compared with Valacyclovir on Genital HSV-2 Shedding in Patients with Frequent Recurrences: a Randomized Clinical Trial. *Jama,* 316**,** 2495-2503.

Wald, A., Zeh, J., Selke, S., ashley, R. L. & Corey, L. 1995. Virologic Characteristics of Subclinical and Symptomatic Genital Herpes Infections. *N Engl J Med,* 333**,** 770-5.

Välimaa, H., Seppänen, M. & Hukkanen, V. 2013. [Herpes Simplex]. *Duodecim,* 129**,** 31-40.

Van Lint, A. L., Murawski, M. R., Goodbody, R. E., Severa, M., Fitzgerald, K. A., Finberg, R. W., Knipe, D. M. & Kurt-Jones, E. A. 2010. Herpes Simplex Virus Immediate-Early ICP0 Protein Inhibits Toll-Like Receptor 2-Dependent Inflammatory Responses and NF-Kappab Signaling. *J Virol,* 84**,** 10802-11.

van Velzen, M., Van De Vijver, D. A., Van Loenen, F. B., Osterhaus, A. D., Remeijer, L. & Verjans, G. M. 2013. Acyclovir Prophylaxis Predisposes to Antiviral-Resistant Recurrent Herpetic Keratitis. *J Infect Dis,* 208**,** 1359-65.

Wang, J. & Quake, S. R. 2014. RNA-Guided Endonuclease Provides a Therapeutic Strategy to Cure Latent Herpesviridae Infection. *Proc Natl Acad Sci U S A,* 111**,** 13157-62.

Wang, L., Chen, X., Zhou, X., Roizman, B. & Zhou, G. G. 2018. miRNAs Targeting ICP4 and Delivered to Susceptible Cells in Exosomes Block HSV-1 Replication in a Dose-Dependent Manner. *Mol ther,* 26**,** 1032-1039.

Weir, H. M., Calder, J. M. & Stow, N. D. 1989. Binding of the Herpes Simplex Virus Type 1 UL9 Gene Product to an Origin of Viral DNA Replication. *Nucleic Acids Res,* 17**,** 1409-25.

Weir, H. M. & Stow, N. D. 1990. Two Binding Sites for the Herpes Simplex Virus Type 1 UL9 Protein Are Required for Efficient Activity of the OriS Replication Origin. *J Gen Virol,* 71 (Pt 6)**,** 1379-85.

Weller, S. K. & Coen, D. M. 2012. Herpes Simplex Viruses: Mechanisms of DNA Replication. *Cold Spring Harb Perspect Biol,* 4**,** A013011.

Verpooten, D., Ma, Y., Hou, S., Yan, Z. & He, B. 2009. Control of TANK-Binding Kinase 1-Mediated Signaling by the Gamma(1)34.5 Protein of Herpes Simplex Virus 1. *J Biol Chem,* 284**,** 1097-105.

Whitley, R. J. 2006. Herpes Simplex Encephalitis: Adolescents and Adults. *Antiviral Res,* 71**,** 141-8.

WHO. 2017. *Herpes Simplex Virus* [Online]. Available at: Https://www.who.int/News-Room/Fact-Sheets/Detail/Herpes-Simplex-Virus [Accessed 09.03.2022].

Wilhelmus, K. R. 2015. Antiviral Treatment and Other Therapeutic Interventions for Herpes Simplex Virus Epithelial Keratitis. *Cochrane Database Syst Rev,* 1**,** Cd002898.

Wilhelmus, K. R., Gee, L., Hauck, W. W., Kurinij, N., Dawson, C. R., Jones, D. B., Barron, B. A., Kaufman, H. E., Sugar, J. & Hyndiuk, R. A. 1994. Herpetic Eye Disease Study. A Controlled Trial of Topical Corticosteroids for Herpes Simplex Stromal Keratitis. *Ophthalmology,* 101**,** 1883-95; Discussion 1895-6.

Visciano, M. L., Mahant, A. M., Pierce, C., Hunte, R. & Herold, B. C. 2021. Antibodies Elicited in Response to a Single Cycle Glycoprotein D Deletion Viral Vaccine Candidate Bind C1q and Activate Complement Mediated Neutralization and Cytolysis. *Viruses,* 13.

Volkov, A. A., Kruglova, N. S., Meschaninova, M. I., Venyaminova, A. G., Zenkova, M. A., Vlassov, V. V. & Chernolovskaya, E. L. 2009. Selective Protection of Nuclease-Sensitive Sites in siRNA Prolongs Silencing Effect. *Oligonucleotides,* 19**,** 191-202.

Wu, C. A., Nelson, N. J., Mcgeoch, D. J. & Challberg, M. D. 1988. Identification of Herpes Simplex Virus Type 1 Genes Required for Origin-Dependent DNA Synthesis. *J Virol,* 62**,** 435-43.

Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., Hu, Y., Tao, Z. W., Tian, J. H., Pei, Y. Y., Yuan, M. L., Zhang, Y. L., Dai, F. H., Liu, Y., Wang, Q. M., Zheng, J. J., Xu, L., Holmes, E. C. & Zhang, Y. Z. 2020. A New Coronavirus associated with Human Respiratory Disease in China. *Nature,* 579**,** 265-269.

Xing, J., Ni, L., Wang, S., Wang, K., Lin, R. & Zheng, C. 2013. Herpes Simplex Virus 1-Encoded Tegument Protein VP16 Abrogates the Production of Beta Interferon (IFN) by Inhibiting NF-Kb Activation and Blocking IFN Regulatory Factor 3 to Recruit its Coactivator CBP. *J Virol,* 87**,** 9788-801.

Xu, F., Schillinger, J. A., Sternberg, M. R., Johnson, R. E., Lee, F. K., Nahmias, A. J. & Markowitz, L. E. 2002. Seroprevalence and Coinfection with Herpes Simplex Virus Type 1 and Type 2 in the United States, 1988-1994. *J Infect Dis,* 185**,** 1019-24.

Yahya, M., Rulli, M., toivonen, L., Waris, M. & Peltola, V. 2017. Detection of Host Response to Viral Respiratory Infection by Measurement of Messenger RNA for MxA, Trim21, and Viperin in Nasal Swabs. *J Infect Dis,* 216**,** 1099-1103.

Ye, G. J., Vaughan, K. T., Vallee, R. B. & Roizman, B. 2000. the Herpes Simplex Virus 1 U(L)34 Protein Interacts with a Cytoplasmic Dynein Intermediate Chain and Targets Nuclear Membrane. *J Virol,* 74**,** 1355-63.

Yin, D., Ling, S., Wang, D., Dai, Y., Jiang, H., Zhou, X., Paludan, S. R., Hong, J. & Cai, Y. 2021. Targeting Herpes Simplex Virus with CRISPR-Cas9 Cures Herpetic Stromal Keratitis in Mice. *Nat Biotechnol,* 39**,** 567-577.

Yuan, S., Wang, J., Zhu, D., Wang, N., Gao, Q., Chen, W., Tang, H., Zhang, X., Liu, H., Rao, Z. & Wang, X. 2018. Cryo-Em Structure of a Herpesvirus Capsid at 3.1 Å. *Science,* 360.

Yuen, M. F., Heo, J., Jang, J. W., Yoon, J. H., Kweon, Y. O., Park, S. J., Tami, Y., You, S., Yates, P., Tao, Y., Cremer, J., Campbell, F., Elston, R., Theodore, D., Paff, M., Bennett, C. F. & Kwoh, T. J. 2021. Safety, Tolerability and Antiviral Activity of the Antisense Oligonucleotide Bepirovirsen in Patients with Chronic Hepatitis B: a Phase 2 Randomized Controlled Trial. *Nat Med,* 27**,** 1725-1734.

Zhang, S. Y., Jouanguy, E., Ugolini, S., Smahi, A., Elain, G., Romero, P., Segal, D., Sancho-Shimizu, V., Lorenzo, L., Puel, A., Picard, C., Chapgier, A., Plancoulaine, S., Titeux, M., Cognet, C., Von Bernuth, H., Ku, C. L., Casrouge, A., Zhang, X. X., Barreiro, L., Leonard, J., Hamilton, C., Lebon, P., Heron, B., Vallee, L., Quintana-Murci, L., Hovnanian, A., Rozenberg, F., Vivier, E., Geissmann, F., Tardieu, M., Abel, L. & Casanova, J. L. 2007. TLR3 Deficiency in Patients with Herpes Simplex Encephalitis. *Science,* 317**,** 1522-7.

Ziegler, T., Waris, M., Rautiainen, M. & Arstila, P. 1988. Herpes Simplex Virus Detection by Macroscopic Reading After Overnight Incubation and Immunoperoxidase Staining. *J Clin Microbiol,* 26**,** 2013-7.

Zuhair, M., Smit, G. S. A., Wallis, G., Jabbar, F., Smith, C., Devleesschauwer, B. & Griffiths, P. 2019. Estimation of the Worldwide Seroprevalence of Cytomegalovirus: a Systematic Review and Meta-Analysis. *Rev Med Virol,* 29**,** E2034.

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