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Enzymatically synthesized 2′-fluoro-modified Dicer-substrate siRNA swarms against herpes simplex virus demonstrate enhanced antiviral efficacy and low cytotoxicity

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ARTICLE INFO

Keywords:
Antiviral
Herpes simplex virus
siRNA
Innate immunity
Fluoro-modification
Bacteriophage phi6 RNA-dependent RNA polymerase

ABSTRACT

Chemical modifications of small interfering (si)RNAs are used to enhance their stability and potency, and to reduce possible off-target effects, including immunogenicity. We have earlier introduced highly effective anti-viral siRNA swarms against herpes simplex virus (HSV), targeting 653 bp of the essential UL29 viral gene. Here, we report a method for enzymatic production and antiviral use of 2′-fluoro-modified siRNA swarms. Utilizing the RNA-dependent RNA polymerase from bacteriophage phi6, we produced 2′-F-siRNA swarms containing either all or a fraction of modified adenosine, cytidine or uridine residues in the antisense strand of the UL29 target. The siRNA containing modified pyrimidines demonstrated high resistance to RNase A and the antiviral potency of all the UL29-specific 2′-F-siRNA swarms was 100-fold in comparison with the unmodified counterpart, without additional cytotoxicity. Modest stimulation of innate immunity signaling, including induced expression of both type I and type III interferons, as well as interferon-stimulated gene 54, by 2′-F-cytidine and 2′-F-uridine modified siRNA swarms occurred at early time points after transfection while the 2′-F-adenosine-containing siRNA was similar to the unmodified antiviral siRNA swarm in this respect. The antiviral efficacy of the 2′-F-siRNA swarms and the elicited cellular innate responses did not correlate suggesting that innate immunity pathways do not significantly contribute to the observed enhanced antiviral activity of the modified siRNAs. The results support further applications of enzymatically produced siRNA molecules with incorporated adenosine nucleotides, carrying fluoro-modification on ribose C2′ position, for further antiviral studies in vitro and in vivo.

1. Introduction

RNA interference (RNAi) is a natural antiviral defense mechanism in plants, fungi, invertebrates, and, under specific conditions, in mammals (Guo et al., 2019; Schuster et al., 2019). Antiviral RNAi comes about via a sequence-specific binding of small interfering RNAs (siRNAs) to viral mRNAs or genomic RNAs, resulting in their degradation or translational repression. In general, siRNA is prone to fast degradation with RNases, and the delivery to the target cells might be difficult. A number of chemical modifications introduced to different positions in the siRNA (sense, antisense, or both strands) have been tested to improve siRNA stability and reduce off-target activities (Braasch et al., 2003; Choung et al., 2006; Czauderna et al., 2003; Fedorov et al., 2006; Watts et al., 2008; Hassler et al., 2018).

Instead of chemical synthesis and post-synthesis hybridization of RNA oligonucleotides, we produce high-quality molecules for RNAi using viral RNA polymerases and recombinant Giardia Dicer enzyme (Levanova and Poranen, 2018). This results in a swarm of 25-bp-long...
In the enzymatically synthesized siRNA swarms, each individual siRNA species is present only in low concentrations, diluting out the potential Dicer, which enhances the RNAi potency and efficacy (Kim et al., 2005). The same protocol was used to produce non-specific unmodified control siRNA swarms from a 413 bp long sequence of Escherichia coli lacI gene in pET32b vector using primers: Fwd_pET32b_1581: 5′-TAATACGACTCACTATAGGGCGCAACCTTGCAGATCAAT-3′ and Rev_pET32b_1983: 5′-GGAAAAAATTGCTGTATCCCATAC-3′. As confirmed by BLASTn, the selected control sequence has no significant similarities with human, herpesviral or mouse transcriptomes. The 88 bp dsRNA was produced as described previously (Jiang et al., 2011).

2.2. RNA stability

Generated siRNAs were treated with RNase A (#EN0531; Thermo Fisher) under the conditions suitable for dsRNA degradation: 1 μg siRNA was incubated with 0.1 μg of RNase A in 20 μl of 0.1 × SSC buffer (20 × SSC: 3 M sodium chloride + 0.3 M sodium citrate in water, pH 7.0) at room temperature for 15 min. The reactions were stopped by addition of 2.5 μl of 10 × loading dye [50% glycerol, 40 mM EDTA pH 8.0, 0.1% bromophenol blue (w/v), 0.1% xylene cyanol] and immediately frozen at −70 °C. The reaction products and equivalent amounts of unmodified siRNA were analyzed by electrophoresis on a 2.5% (w/v) agarose gel containing 0.2 μg/ml EtBr in Tris-acetate-EDTA (TAE) buffer at 90 V, 200 mA, 1 h. The RNAs were visualized with Chemidoc Touch Imaging System (Bio-Rad) and the band intensities measured using Fiji (Schindelin et al., 2012). The relative amount of degraded siRNA was calculated according to the formula: a%=([S]t/[S]0)×100/([S]u/[S]0), where [S]t is a density of RNA-treated siRNA and [S]u is a density of the equivalent amount of untreated sample.

2.3. Cells and virus

An astrocytoma-glioma cell line (U373MG) obtained originally from ATCC (Manassas, VA) was used. The line has been subsequently reclassified as U251 human glioblastoma, but referred here as U373MG to ensure continuity with our previous studies (Paavilainen et al., 2015, 2016; Romanovskaya et al., 2012). U373MG cells were maintained in DMEM with 10% heat inactivated fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C, 5% CO2. Vero cells (ATCC), used for plaque titration, were maintained in M199 medium with 5% FBS.

Green fluorescent protein (GFP)-expressing HSV-1 strain, HSV-1 (17–)Lox-PmCMV-GFP (abbreviated here as HSV-1-GFP), originally received from prof. Beate Sodeik (MHH Hannover Medical School, Germany), was used for antiviral studies (Snijder et al., 2012; Mattila et al., 2015) and propagated as previously described (Romanovskaya et al., 2012).

2.4. Transfection

The U373MG cells were transfected on 96-well plates with either modified or unmodified siRNA swarms, 88 bp dsRNA or water (mock transfection) using Lipofectamine RNAiMAX (#13778-150; Invitrogen, Carlsbad, CA) according to the manufacturer’s forward transfection protocol (Romanovskaya et al., 2012). Depending on the experiment, the cells were transfected with 1–10 pmol of RNA per well, which is equivalent to a concentration of 10–100 nM. All experiments were repeated at least twice with three or more biological replicates each.

2.5. Cellular viability

Cellular viabilities in siRNA treatments were assessed 48 h post transfection (hpt) with CellTiter-Glo (#G7571; Promega, Madison, WI) luminescent assay (Romanovskaya et al., 2012; Turunen et al., 2016). The luminescence was quantified with VICTOR Nivo Multimode Plate Reader (PerkinElmer, Waltham, MA). Viability of more than 80% compared to mock- and untreated cells was considered acceptable.
2.6. Quantitation of innate immunity responses by RT-qPCR

The total cellular RNA was extracted from siRNA-treated U373MG cells at 8, 24, and 48 hpt with TRI Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The RNA was processed to complementary DNA using RevertAid H Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) with random hexamer primers (Thermo Fisher Scientific) after treatment with DNAse (Thermo Fisher Scientific) (Romanovskaya et al., 2012; Paavilainen et al., 2015, 2016). Quantitative PCR (qPCR) was performed with Rotor-Gene Q, as previously described (Nygardas et al., 2011). The GAPDH housekeeping mRNA values of the corresponding sample were used for normalization of mRNA expression levels. The mRNA expression of interferon beta (IFN-β) (Peri et al., 2008), lambda 1 (IL-29; IFN-λ1) (Paavilainen et al., 2016), interferon stimulated gene 54 (ISG54) (Romanovskaya et al., 2012), and GAPDH (Nygardas et al., 2009) was quantified using previously published primer sequences.

2.7. Quantitation of antiviral efficacy

The RNA- or control-treated cells were challenged with a clinically relevant infectious dose of HSV-1-GFP at 1000 plaque forming units (pfu) in 100 μl per well [multiplicity of infection (MOI) ≈ 0.06] at 4 hpt in 96-well plates as before (Paavilainen et al., 2016). At 44 h post infection (hpi), the culture supernatant was collected, and the viral titer was determined by plaque titration on Vero cells in 96-well plates.

2.8. Live-cell imaging

The HSV-1-GFP strain enabled fluorescent imaging of the virus infection and thus also of the antiviral effect. The live cell imaging was performed 48 hpt with EVOS Auto FL (Thermo Fisher Scientific) with the whole plate scan program, keeping the imaging parameters equal throughout the scan.

2.9. Statistical analysis

Statistical analysis was conducted with SPSS Statistics 26.0.0.0. (IBM, Armonk, NY). Statistical significances were calculated with Mann-Whitney’s non-parametric U test comparing two independent groups.
3. Results

3.1. Phi6 RdRp can use 2'-F-dNTPs as substrate to produce 2'-F-dsRNA for dicer reaction

We tested the capability of phi6 RdRp to utilize 2'-F-dNTPs and 2’-OMe-NTPs in the dsRNA synthesis. HSV UL29 gene-specific siRNA, produced using T7 DdRp, was used as a template for the phi6 RdRp-catalyzed dsRNA synthesis. Initially, the reactions were done by replacing one of the canonical NTPs in the reaction mixture with the corresponding modified NTP. Although dsRNA synthesis was not effective in the presence of 2’-OMe-modified NTPs (Fig. S1) and the quality of the dsRNA product was somewhat compromized in the presence of 2’-F-dGTP (Fig. S2A), phi6 RdRp was able to produce full-length dsRNA products using 2’-F-dATP, 2’-F-dUTP, and 2’-F-dCTP substrates (Fig. 1A), suggesting that the 113 adenosines, 128 uridines, and 185 cytidines in the antisense strand of the HSV UL29 target sequence can potentially be replaced with their fluoro-modified counterparts. Wild-type phi6 RdRp and a mutant RdRp containing substitution K–A at position 147 (K147A) were initially tested for dsRNA production. The K147A RdRp consistently provided higher dsRNA yields (Fig. S2B) and was therefore used for the subsequent siRNA production. Complete replacement of a canonical NTP with the corresponding 2’-F-dNTP resulted in slightly decreased yields, which was especially pronounced when all three NTPs (ATP, UTP and CTP) carried the modification (Fig. 1A). Therefore, to produce siRNA swarms for the cellular experiments we used 2’-F-dATP, 2’-F-dCTP, or 2’-F-dUTP, but not their mixture. Furthermore, we set up a dsRNA synthesis reaction in which only a fraction (10%) of a given NTP was fluoro-modified (Fig. S2). The mass spectrometry analysis confirmed that the “10% modified” dsRNA products contained 2’-fluoro-modified nucleotides (Fig. S3). Furthermore, the Giardia Dicer could process the produced UL29-2’-F-dsRNA into a swarm of Dicer-substrate 2’-F-siRNAs (Fig. 1B).

3.2. Stability, cytotoxicity and antiviral efficacy of the 2’-F-siRNA swarms

3.2.1. Stability of the 2’-F-siRNA swarms

RNase A treatment in low salinity conditions was performed to assess sensitivity of the 2’-F-siRNA swarms to RNase. Under the conditions used, about 50% of the unmodified siRNA was degraded (Fig. 1C), whereas fluoro-modifications in the uridine and cytidine residues significantly enhanced the stability of the siRNAs.

3.2.2. Modified 2’-F-siRNA swarms are well tolerated in vitro

Cellular toxicity of the 2’-F-siRNA swarms was assessed using U373MG cells in 96-well plates. Cells were transfected with each of the HSV-specific UL29-gene derived-2’-F-siRNA swarms, an unmodified UL29-siRNA swarm, a non-specific siRNA swarm derived from E. coli lacI gene, a cytotoxic 88-bp-long control dsRNA, or water. The viability of the cells was assessed 48 hpt with luminescent assay (Fig. 2). Transfections with the 2’-F-siRNA swarms resulted in similar levels of cellular viability to that with unmodified UL29-siRNA swarms, with the exception of the fully 2’-F-dCTP modified swarm, which was significantly less tolerated than the unmodified siRNA swarm at 50 and 100 nM. Nevertheless, all the 2’-F-siRNA swarms were well tolerated, with relative cellular viability consistently higher than 80% at doses relevant for antiviral applications. Hence, 2’-F-modifications had no effect on tolerability of the siRNA swarms in the studied concentration range. The UL29-targeting siRNA swarms did not display increased cytotoxicity in comparison to the non-specific siRNA swarm, targeting a non-relevant bacterial sequence. The 88 bp dsRNA used as a toxic control resulted in a clear decrease in cell viability at 10 nM concentration (Fig. 2). The cytotoxicity profile of the modified siRNA swarms was confirmed with non-cancerous human corneal epithelial cells, in which only the fully 2’-F-dCTP modified swarm was significantly less tolerated than the unmodified siRNA swarm (Fig. S4).

3.2.3. Fluoro-modifications increase antiviral efficacy of enzymatically produced UL29-siRNA swarms

The antiviral efficacy of the UL29-2’-F-siRNA swarms was quantified at 48 hpt by plaque titration from culture supernatants of HSV-1-GFP-
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A

\[ \text{Viral titer (pfu/ml)} \]

\[
\begin{array}{cccc}
100\% & F-A/C/U & & \\
10\% & F-A/C/U & & \\
\text{unmodified} & & & \\
\text{non-specific} & & & \\
\text{swarm} & & & \\
\text{treatment} & & & \\
\text{mock-} & & & \\
\text{or} & & & \\
\text{untreated} & & & \\
\end{array}
\]

B

\[ \text{Fold change of titer compared to untreated cells} \]

\[
\begin{array}{cccc}
10\% & F-A & & \\
10\% & F-A & & \\
10\% & F-C & & \\
10\% & F-C & & \\
10\% & F-U & & \\
100\% & F-U & & \\
\text{unmodified} & & & \\
\text{non-specific} & & & \\
\text{swarm} & & & \\
\end{array}
\]

C

\[ \text{Fold change of titer compared to untreated cells} \]

\[
\begin{array}{cccc}
2'F & \text{modified} & & \\
\text{UL29-swarm} & & & \\
\text{treatment} & & & \\
\text{mock-} & & & \\
\text{treated} & & & \\
\end{array}
\]

D

10\% F-A

10\% F-C

10\% F-U

Unmodified

Mock-treated

100\% F-A

100\% F-C

100\% F-U

Non-specific

Untreated

1000 \mu m

(caption on next page)
infected U373MG cells. Before infection, cells were treated with HSV-targeted modified or unmodified UL29-siRNA swarms, a non-specific siRNA swarm, water (mock-treated), or left untreated (Fig. 3). After treatment with the unmethylated UL29 siRNA swarm, the viral titer was reduced at least three orders of magnitude compared to the untreated targeted modified or unmodified UL29-siRNA swarms, a non-specific swarm after treatment with each of the UL29-2′A.A. Levanova et al.

The efficacy of the HSV-targeted siRNA swarms against HSV-1-GFP infection on U373MG cells was visualized by living cell fluorescent imaging at 48 hpt (at 44 hpi; Fig. 3 D). The virus-derived GFP signal was not detected using standard imaging parameters. The diamonds illustrate the individual samples. Significant changes against the unmodified UL29-specific siRNA are marked with asterisks (*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ns: non-significant). (B, C) Representative fluorescent images of the HSV-1-GFP-infected cells with the indicated siRNA swarm or control treatments were taken at 48 hpt. The scale bar represents 1000 μm.

3.3. Induction of innate immunity responses by 2′-F-siRNA swarms

Consistent with earlier research, transfection of 88 bp dsRNA, an immunostimulatory and cytotoxic molecule (Jiang et al., 2011), induced IFN-β, IL-29 and ISG54 expression in the transfected cells (Fig. 4) (Romanovskaya et al., 2012; Paavilainen et al., 2015). In comparison with untreated cells, the unmethylated UL29 siRNA swarm induced modest ISG54 and IL-29 responses (Fig. 4B and C) but did not significantly alter IFN-β expression (Fig. 4A) at any point time. Notably, only the ISG54 levels at 8 hpt differed significantly between the unmethylated UL29 siRNA and transfection reagent alone (mock-treated) (Fig. 4C), indicating that the transfection reagent causes the majority of the responses in comparison with the untreated cells. 2′-F-dUMP- and 2′-F-dCMP-siRNAs induced higher IFN-β, IL-29 and ISG54 expression compared to unmethylated UL29-siRNA swarm, particularly at the earliest time point (Fig. 4). Innate immunity signaling induced by 2′-F-dAMP-siRNA swarms and that by the unmethylated UL29 siRNA were at equal levels, or even lower. Overall, the induction of interferon signaling by modified or unmethylated siRNA swarms was not extensive (only ca. 10-fold compared to mock- and untreated cells).

4. Discussion

We have previously reported a novel enzymatic approach to synthesize large pools of siRNA molecules, which we refer to as siRNA swarms. The antiviral siRNA swarms directed against the current target of choice, the UL29 gene of HSV-1, have previously demonstrated their efficacy against circulating pathogenic HSV strains in vitro and corneal infection in vivo, unveiling their potential as antiviral drugs. In the current study, we pursued the modification of the RNAs using nucleotides with 2′-modified ribose, in order to further enhance the antiviral potency and stability of the siRNA preparations. Eventually, we are able to report successful enzymatic incorporation of 2′-F-dNTP nucleotides to biologically active dsRNA molecules. All 2′-F-siRNA swarms were well tolerated and highly effective against HSV-1 in vitro. The observed enhanced antiviral efficacy of the 2′-F-siRNA swarms was unrelated to the elicited cellular innate immunity responses, proving the sequence specificity of the modified siRNAs.

We produced siRNA swarms, in which either all or a fraction of adenosines, cytidines or uridines were replaced by 2′-F-dNMPs. Replacement of a 2′-OH group of ribose with a fluorine has been shown to stabilize RNA molecules against nucleases (Monia et al., 1993). SiRNAs are primarily degraded by RNase A (Turner et al., 2007), which allows enzymatic production of perfectly duplexed dsRNAs without the often error-prone post-synthesis hybridization. Similarly, to other wild-type bacteriophage polymerases (Sousa and Padilla, 1995; Zhu et al., 2015), phi6 RdRp did not tolerate 2′-OMe modifications (Fig. S2). All of the modified siRNA swarms were non-toxic for U373MG cells at the relevant antiviral concentrations. The findings were reproducible in human corneal epithelial cells (Fig. S4), which represent another potential target tissue for antiviral therapy of diseases caused by HSV-1. Furthermore, modified swarms had higher antiviral activity than the unmethylated swarm, as demonstrated by the significant reduction of viral titer in plaque assays. Notably, there was no difference between siRNA swarms containing only a fraction of modified dNMPs and those containing all modified dCMPs, dUMPs, or dAMPs in the antisense strand.
The antisense strand of the modified siRNA swarms used was fully (100%) or partially (10%) modified containing either 2'-F-dAMP or 2'-D-dCMP as the modified nucleotide (10% or 100% F-A, F-C or F-U, respectively). The expression levels of interferon beta (IFN-β; panel A), interferon lambda-1 (IFN-λ1, IL-29; panel B) and interferon-stimulated gene 54 (ISG54; panel C) were quantified at 8 hpt (black bars), 24 hpt (dark grey bars) or 48 hpt (light grey bars) by RT-qPCR. The expression levels, normalized to housekeeping gene GAPDH, are presented on a logarithmic scale. The means and standard deviations, from two separate experiments with at least four repeat samples, are presented. Significantly higher induction of IFNs and ISGs in comparison to the unmodified UL29-specific swarm treatment is marked with asterisks (*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001) and significantly lower induction with #: p ≤ 0.05, ###: p ≤ 0.01, ####: p ≤ 0.001). If no p-value is shown, the difference to the unmodified UL29-specific swarm is not significant.

![Fig. 4. Innate immunity responses induced by 2'-F-siRNA swarms.](image)

A

B

C

The antiviral effect of siRNAs containing only a fraction of modified dUMPs was not statistically different from the unmodified siRNA swarm. Interestingly, a higher stability to nucleases of siRNA swarms containing 2'-F-dUMPs or 2'-D-dCMPs did not translate into higher antiviral efficacy than those with 2'-F-dAMPs.

The immunostimulatory potential of 2'-F-dAMP-siRNAs resembled that of the unmodified siRNA swarm and was thus minimal. At the early time point (8 hpt), 2'-F-dUMP-siRNA and 2'-D-dCMP-siRNA swarms induced slightly higher expression of IFN-β, IL-29, and ISG54 than the unmodified siRNA, implying that their antiviral activity at earlier time points might be partly non-specific and related to the stimulation of the innate immune response. However, 2'-F-dAMP-siRNA swarms demonstrated enhanced antiviral efficacy in comparison with the unmodified swarm, but similar immunostimulatory activity. Therefore, antiviral 2'-F-dAMP-siRNA swarm is a promising candidate for further in vivo studies with HSV.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

Dr. Ian MacRae is thanked for the generous gift of pFB-GiDcr plasmid used for the production of Giardia Dicer. Tanja Westerholm, Ritva Kajander, and Marja-Leena Mattila are thanked for technical assistance. Prof. Arto Urtti (University of Helsinki and University of Eastern Finland, Finland) is acknowledged for providing the corneal epithelial cells.

This work benefited from access to the Instruct-HILIFE Biocomplex unit, a FINstruct and Instruct-ERIC center and a member of Biocenter Finland. The use of the facilities and expertise of Metabo-Hilife Viikki Metabolomics Unit, member of Biocenter Finland, is gratefully acknowledged. The work was financially supported by the Jane and Aatos Erkko Foundation, grant #170046, and by Sigrid Juselius Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.antiviral.2020.104916](https://doi.org/10.1016/j.antiviral.2020.104916).

References

Braasch, D.A., Jensen, S., Liu, Y., Kaur, K., Azar, K., White, M.A., Corey, D.R., 2003. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry 42, 7967–7975. [https://doi.org/10.1021/bi0349774](https://doi.org/10.1021/bi0349774).

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, 919–927. [https://doi.org/10.1016/j.bbrc.2006.02.049](https://doi.org/10.1016/j.bbrc.2006.02.049).

Cuchillo, C.M., Nogues, M.V., Raines, R.T., 2011. Bovine pancreatic ribonuclease: fifty years of the first enzymatic reaction mechanism. Biochemistry 50, 7835–7841. [https://doi.org/10.1021/bi201075b](https://doi.org/10.1021/bi201075b).

Cauderna, F., Fechtner, M., Dames, S., Aygun, H., Klippel, A., Pronk, G.J., Giese, K., Kaufmann, J., 2003. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. 31, 2705–2716. [https://doi.org/10.1093/nar/gkg398](https://doi.org/10.1093/nar/gkg398).

Fedorov, Y., Anderson, E.M., Birmingham, A., Reynolds, A., Karpilow, J., Robinson, K., Leake, D., Marshall, W.S., Khvorova, A., 2006. Off-target effects of siRNA can induce toxic phenotype. RNA 12, 1188–1196. [https://doi.org/10.1261/rna.26189](https://doi.org/10.1261/rna.26189).

Guo, Z., Li, Y., Ding, S.W., 2019. Small RNA-based antimicrobial immunity. Nat. Rev. Immunol. 19, 31–44. [https://doi.org/10.1038/s41577-018-0071-x](https://doi.org/10.1038/s41577-018-0071-x).

Hassler, M.R., Turalov, A.A., Alterman, J.F., Harastzi, R.A., Colen, A.H., Osborn, M.F., Echeverria, D., Nikan, M., Solomon, W.E., Roux, L., Godinho, B., Davis, S.M., Morrissey, D.V., Zamore, P.D., Karumanchi, S.A., Moore, M.J., Aronin, N., Khvorova, A., 2018. Comparison of partially and fully chemically-modified siRNA in conjugate-mediated delivery in vivo. Nucleic Acids Res. 46, 2185–2196. [https://doi.org/10.1093/nar/gky077](https://doi.org/10.1093/nar/gky077).
Jackson, A.L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J.M., Lim, L., Karpillow, J., Nichols, K., Marshall, W., Khvorova, A., Linsley, P.S., 2006. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA 12, 1197–1205. https://doi.org/10.1261/rna.30706c.

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, 1713–1721. https://doi.org/10.4049/jimmunol.1100361.

Jiang, M., Osterlund, 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 monocytic-derived macrophages and dendritic cells. J. Virol. 93, e01916–e01918. https://doi.org/10.1128/JVI.01916-18.

Kim, D.H., Behlke, M.A., Rose, S.D., Chang, M.S., Choi, S., Rossi, J.J., 2005. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat. Biotechnol. 23, 222–226. https://doi.org/10.1038/nbt1051.

Levanova, A., Poranen, M.M., 2018. RNA interference as a prospective tool for the control of human viral infections. Front. Microbiol. 9, 2151. https://doi.org/10.3389/fmicb.2018.02151.

Macrae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., Doudna, J.A., 2006. Structural basis for double-stranded RNA processing by Dicer. Science 311, 195–198. https://doi.org/10.1126/science.1121630.

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. https://doi.org/10.1099/jgv.0.085013.

Monia, B.P., Gonzalez, C., Lima, W.F., Guinosso, C.J., Mattila, R.K., Harila, K., Kantola, H., Waris, 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, 14514–14522.

Niehl, A., Soininen, M., Poranen, M.M., Heinlein, M., 2018. Synthetic biology approach for plant protection using dsRNA. Plant Biotechnol. J. 16, 1679–1687. https://doi.org/10.1111/pbi.12904.

Nygardas, M., Voutilainen, H., Lehtinen, J., Romanovskaya, A., Nygardas, M., Bamford, D.H., Poranen, M.M., Hukkanen, V., 2011. Treatment of experimental autoimmune encephalomyelitis in SJL/J mice with a replicative HSV-1 vector expressing interleukin-5. Gene Ther. 18, 646–655. https://doi.org/10.1038/gt.2011.4.

Nygardas, M., Vuorinen, T., Aalto, A.P., Bamford, D.H., Hukkanen, V., 2009. Inhibition of cossackievirus B3 and related enteroviruses by antiviral short interfering RNA pools produced using phi6 RNA-dependent RNA polymerase. J. Gen. Virol. 90, 2468–2473. https://doi.org/10.1099/jgv.0.011328-0.

Paavilainen, H., Lehtinen, J., Romanovskaya, A., Nygardas, 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. https://doi.org/10.1002/jmv.24578.

Paavilainen, H., Romanovskaya, A., Nygardas, M., Bamford, D.H., Poranen, M.M., Hukkanen, V., 2015. Innate responses to small interfering RNA pools inhibiting herpes simplex virus infection in astrocytoid and epithelial cells. Innate Immun. 21, 349–357. https://doi.org/10.1177/1753425914537921.

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. https://doi.org/10.1186/1743-422X-5-140.

Romanovskaya, A., Paavilainen, H., Nygardas, 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. https://doi.org/10.1371/journal. pubs.0051019.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2013.

Schuster, S., Miesen, P., van Rij, R.P., 2019. Antiviral RNAi in insects and mammals: parallels and differences. Viruses 11, 3614. https://doi.org/10.3390/v11121054.

Snijder, B., Sacher, R., Ramo, 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., Balint, G., Schellhaas, M., De Haan, C.A., Marjomaki, V., Hyypia, T., Rottier, P.J., Sodelk, B., Marsh, M., Grunenberg, J., Amara, A., Greber, U., Helenius, A., Pellmann, L., 2012. Single-cell analysis of population context advances RNAi screening at multiple levels. Mol. Syst. Biol. 8, 579. https://doi.org/10.1038/msb.2012.9.

Souza, R., Padilla, R., 1995. A mutant T7 RNA polymerase as a DNA polymerase. EMBO J. 14, 4609–4612.

Turner, J.J., Jones, S.W., Moschos, S.A., Lindsay, M.A., Gait, M.J., 2007. MALDI-TOF mass spectral analysis of siRNA degradation in serum confirms an RNAse A-like activity. Mol. BioSyst. 3, 43–50. https://doi.org/10.1039/B611644B.

Turunen, A., Hukkanen, V., Kulkala, J., Syrjanen, S., 2016. HSV-1 infection modulates the radiosensitivity of a HPV16-positive head and neck cancer cell line. Anticancer Res. 36, 565–574.

Watts, J.K., Deleavey, G.F., Damha, M.J., 2008. Chemically modified siRNA: tools and applications. Drug Discov. Today 13, 842–855.

Zhu, B., Hernandez, A., Tan, M., Wollenhaupt, J., Tabor, S., Richardson, C.C., 2015. Synthesis of 2′-fluoro RNA by SynTh RNA polymerase. Nucleic Acids Res. 43, e94. https://doi.org/10.1093/nar/gkv367.