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STRUCTURE, FUNCTION AND ANTIGENICITY OF A NOVEL SMALL RNA

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ABSTRACT

The phenomenon of RNA interference (RNAi) was discovered in the 1990's when a kind of gene specific silencing was observed in plants and platyhelminthes. Further studies in the field of RNAi yielded the two classes of RNA called short interfering RNA (siRNA) and microRNA (miRNA). These two classes of RNA downregulate gene expression by either cleaving targeted mRNA or by inhibiting the translation of proteins from the mRNA.

These RNA variants have a therapeutic value in regulating gene expression in diseases difficult to treat. The RNA variants are however associated with several problems with regards to the stability of the molecules, the efficiency of the molecules and the immunogenicity of the molecules.

Recently a novel kind of small RNA (sRNA) has been found. This sRNA has the capability of regulating the gene expression of virus associated proteins. Further studies have shown that this sRNA could be modified to target other genes as well. In this study I have further examined this sRNA with regards to what affects its stability, what affects its function and how immunogenic it is compared to conventionally used siRNA and miRNA.

The results from this study show that the structure of the sRNA both affects the stability and the function of the sRNA. By modifying one part of the sRNA it is possible to alter the stability, while altering the other part of the sRNA alters its function. This study also showed that this sRNA is less antigenic than the conventional siRNA and miRNA.

These results indicate that this sRNA could be developed into a novel therapeutic molecule to be used to treat different kinds of diseases. More research on the characteristics of the sRNA is however still needed before it can be used to treat a living vertebrate.

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INTRODUCTION

1. Introduction

1.1 Background

The phenomenon of RNA interference (RNAi) was discovered in the 1990's when a kind of gene specific silencing was discovered in plants and platyhelminthes. These findings were soon followed by the discovery of the same principal mechanism in mammals in the early 2000's. Today these classes of RNA interfering molecules are called short interfering RNA (siRNA) and microRNA (miRNA). (1)

siRNA and miRNA are similar in synthesis and function, but they differ at key aspects. siRNAs are derived from double stranded RNA (dsRNA). The dsRNA is processed by the cytoplasmic enzyme Dicer to form an approximately 22 nucleotide long siRNA. This siRNA is then loaded onto another protein, called Argonaute 2 (AGO2) and forming the so called RNAi-induced silencing complex (RISC). AGO2 then recognizes if the strands in the loaded duplex is completely complementary to each other and cleaves one of the strands, the passenger strand. The remaining guide strand is then used by the RISC to direct AGO2 mediated mRNA cleavage to genetic material fully complementary to the guide strand. When a complementary strand is found, AGO2 cleaves the phosphodiester bond of the targeted mRNA between the tenth and eleventh base relative to the 5' end of the guide strand. This cleaved mRNA is then further degraded by several different exonucleases. (1-3)

miRNA on the other hand typically originate from endogenously created short hairpin transcripts, pri-miRNA. The pri-miRNA is cleaved by the nuclear enzyme Drosha to create a pre-miRNA. The pre-miRNA is transported to the cytoplasm via the exportin 5 nuclear receptor and loaded onto Dicer. As with dsRNA, Dicer also cleaves the pre-miRNA to form the mature miRNA duplex. The strands in the miRNA duplex are however not completely complementary and when the miRNA bind into AGO2, AGO2 does not cleave the passenger strand but instead unwind the strands from each other. The loaded guide strand miRNA is then used by the RISC to block translation and induce mRNA degradation, most often by binding via the so called seed region of the miRNA to the 3' untranslated region of the mRNA. The seed region is a region consisting of nucleotides two to seven relative to the 5' end of the miRNA. Since the seed region is relatively short, it has many complementary sites it can bind to and therefore it can regulate the expression of several different mRNA. (1-3)

INTRODUCTION

Due to the highly specific gene silencing of genes by siRNA, the therapeutic value of siRNA is potentially large. Studies have shown that siRNA has the potential to downregulate specific allelic variants of genes down to single nucleotide polymorphisms. However, miRNA regulate genes only by decapping the mRNA, which means that it does not have the same potency as siRNAs. (1)

One of the major obstacles associated with the use of RNAi therapeutically, is its activating capacity of the immune system. If the RNA species is too long or if it contains specific sequences (PAMPs), the cells will recognize the RNA as a virus and the antiviral defense mechanisms will be activated. dsRNA is recognized by PKR and the Toll like receptor 3 and 9 (TLR3/9), while ssRNA is recognized by the Toll like receptor 7 and 8 (TLR7/8). The TLRs are receptors found on the inside of the endosome and their role is to recognize potential foreign nucleic acids. The receptors are however selective towards GU rich sequences, meaning that this could be exploited in the design of synthetic RNA for therapeutic or experimental purposes. Other motifs could exist, but more research is needed. Recognition by these receptors can also be evaded by 2'-O-methyl modifications of the synthetic RNA. It is also possible to create a methylene bridge between the 2'-O and the 4'-C in the ribose to create a sterically locked ribose, which is not as well recognized by the different TLRs. (1, 3-4)

To assess whether or not a specific RNA initiates an immune response, the levels of the different cytokines and interferons produced can be examined. The TLR signalling pathways are initiated by their corresponding PAMPs. Depending on the activated TLR, different signalling pathways gets activated. One common pathway is however the MyD88-dependent pathway. When the receptor is activated, MyD88 forms a complex with IRAK4, which in turn activates IRAK1. IRAK1 together with TRAF6 activates TAK1. TAK1 is a member of the MAPK family of proteins and activation of this protein leads to activation of the NF-κB and MAPK pathways. These pathways stimulate the synthesis of different proinflammatory cytokines, such as IL-1β and IL-6, as well as IL-12, TNF and different type I interferons. (5-7)

Some TLRs also activate a TRIF dependent pathway in which the TLR activates TRIF. This adaptor protein TRIF recruits TRAF3 and TRAF6. TRAF3 in turn recruits the kinases NEMO, IKKi and TBK1 to be able to phosphorylate IRF3, which in turn induces the expression of genes coding for type I interferons. TRAF6 recruits RIP-1, leading to activation of the TAK1 complex. This complex in turn activates the NF-κB and MAPK pathways. (5-7)

INTRODUCTION

Another problem to overcome with regards to the use of RNAi is the low stability of the naked RNA species, for example naked siRNA has a half life of only a couple of minutes in mammalian plasma. By changing the ribose backbone it is however possible to alter the properties of the RNA, for example to evade endonuclease degradation by modifying the ribose at the 2' end of the RNA. Sterically locking the ribose also works for evading nuclease degradation. (1, 3)

As of lately, a new kind of RNA species has been discovered. It has been named MLP-TSS-sRNA and it was found to originate from the transcriptional start site of the adenovirus Major Late Promoter. This new sRNA was found to contain a m7G-cap by comparing the crosslinking efficacy of unmodified RNA to a nylon membrane compared to the crosslinking efficacy of TAP treated sRNA. The sRNA was later found to be enriched in AGO2/RISC by extracting AGO2/RISC in followed by a second round of immunoprecipitation. By knocking down the enzyme Dicer it was possible to assess that the MLP-TSS-sRNAs are synthesized by a different pathway compared to regular miRNA and other virus associated miRNA. The function of the sRNA was then studied by creating a luciferase reporter construct complementary to the MLP-TSS-sRNA binding site. The expression of the luciferase construct was found to be lower in cells containing MLP-TSS-sRNA. Finally the structure of the MLP-TSS-sRNA was examined. The 3' half of the sRNA was predicted to fold into a secondary structure containing a stabilizing hairpin structure and a 5' single stranded part. This structural organization was tested experimentally by modifying the sequence of the sRNA. When the hairpin structure was disturbed, the half-life of the sRNA was significantly lower compared to the unmodified and stable sRNA. (2)

1.2 Aim

Firstly, through a series of mutations in MLP-TSS-sRNA sequence, I was investigating how the MLP-TSS-sRNA as a single stranded small RNA is protected from rapid RNA degradation in vivo.

Secondly, I assessed how the non-hairpin structure affects the function of the sRNA by making a firefly luciferase construct containing a binding site complementary to the sequence of the non-hairpin structure sequence on the sRNA.

Thirdly, I compared a panel of different conventional sRNAs to the TSS variants with regards to their mmunostimulatory effect by measuring the amount of NF-κβ-activating cytokines released by cells transfected with the different sRNAs.

2. Results

The stability of the sRNA is affected by its hairpin structure

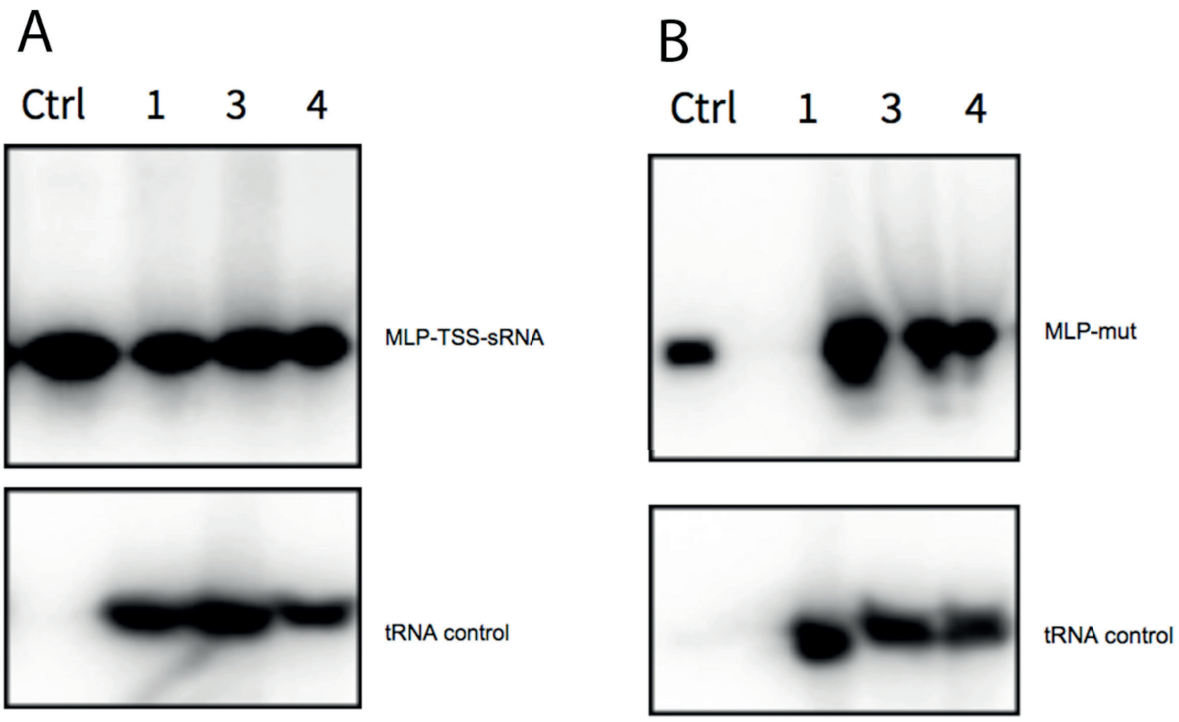

Figure 1. Stability of WT MLP-TSS-sRNA (A) and mut MLP-TSS-sRNA (B) at 1, 3 and 4 days post transfection.

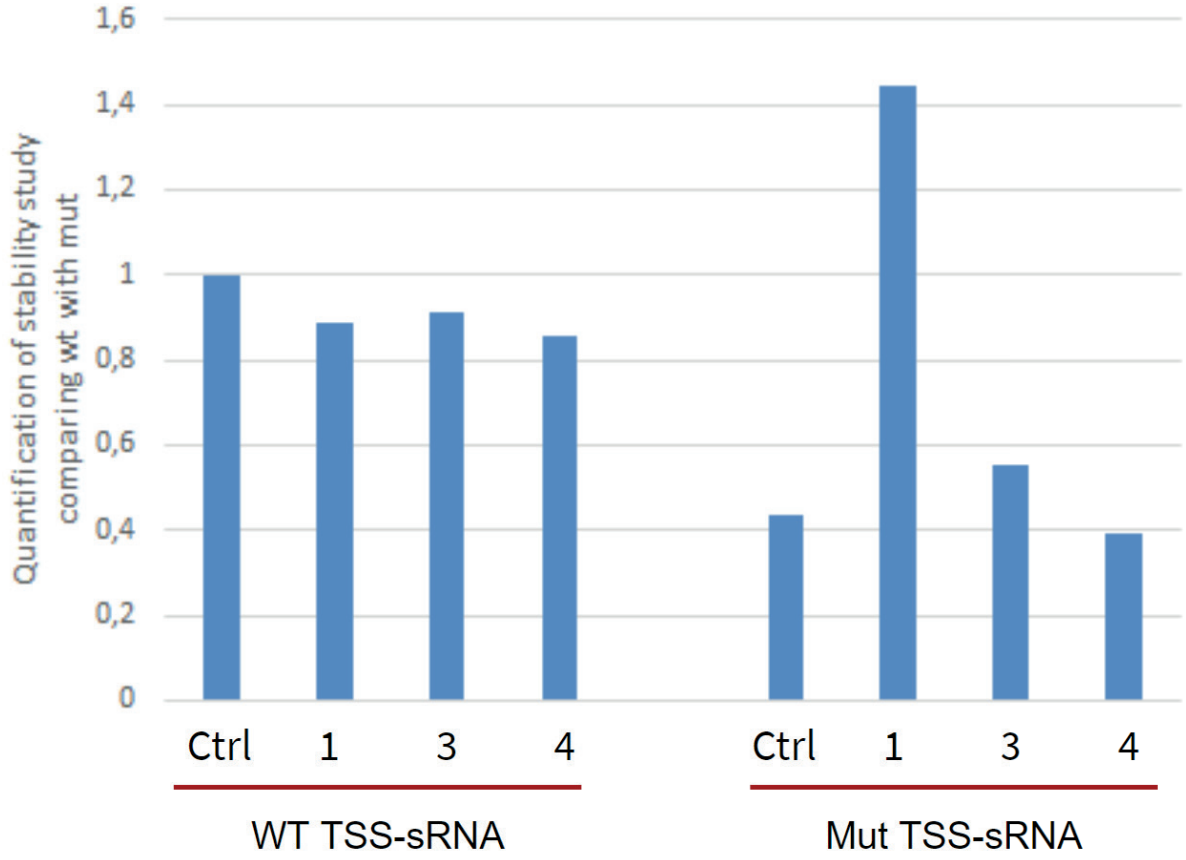

Figure 2. Quantification of northern blot from stability study comparing WT TSS-sRNA and mut TSS-sRNA at days 1, 3 and 4.

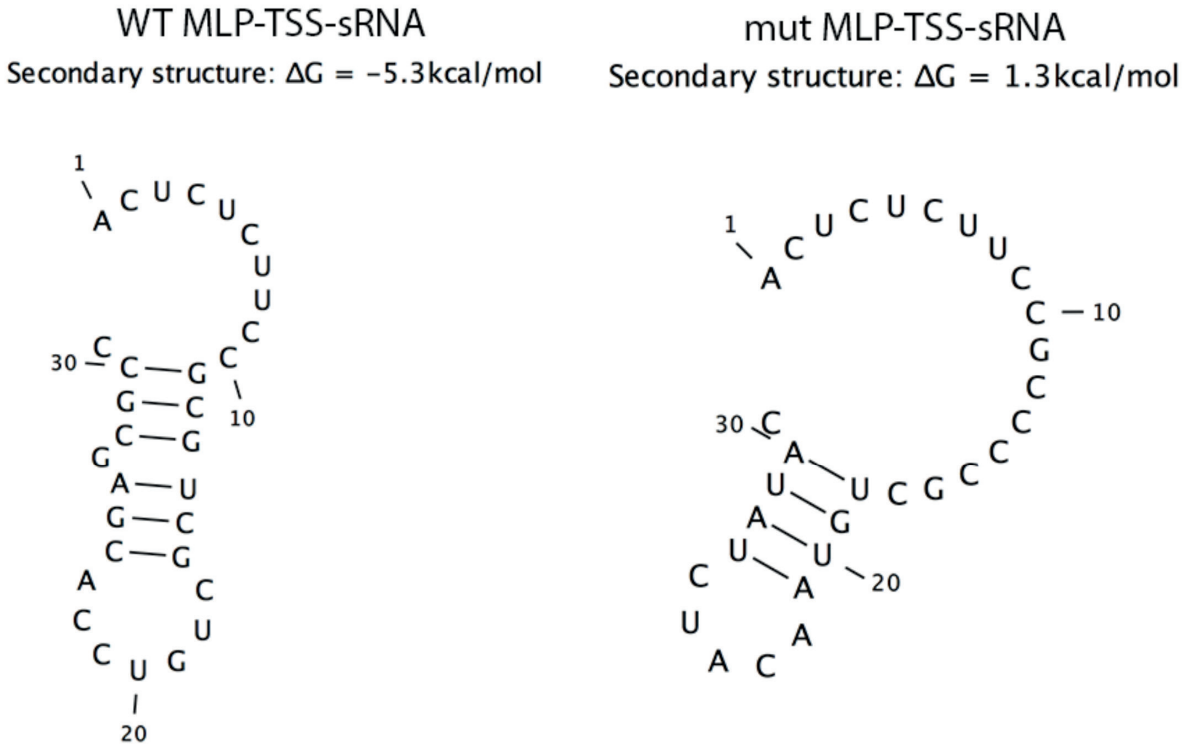

Figure 3. Primary and predicted secondary structure of the WT MLP-TSS-sRNA and the mutated MLP-TSS-sRNA.

To determine what makes the wild type MLP-TSS-sRNA stable, HeLa cells were transfected with different sets of modified variants of the wild type MLP-TSS-sRNA. Cells were then harvested at different timepoints and the stability of the RNA was examined by quantifying the amount of RNA on a northern blot. The first study consisted of comparing the wild type MLP-TSS-sRNA to a mutated version of the same sRNA. The results from this study (Figure 1) show that the wild type MLP-TSS-sRNA show no signs of degradation after four days, whereas the mutated version show degradation after three days. This is in line with the predicted stability of the different RNAs, where the free energy of the wild type is calculated to -5.3 kcal/mol compared to the predicted value of 1.3 kcal/mol (Figure 3).

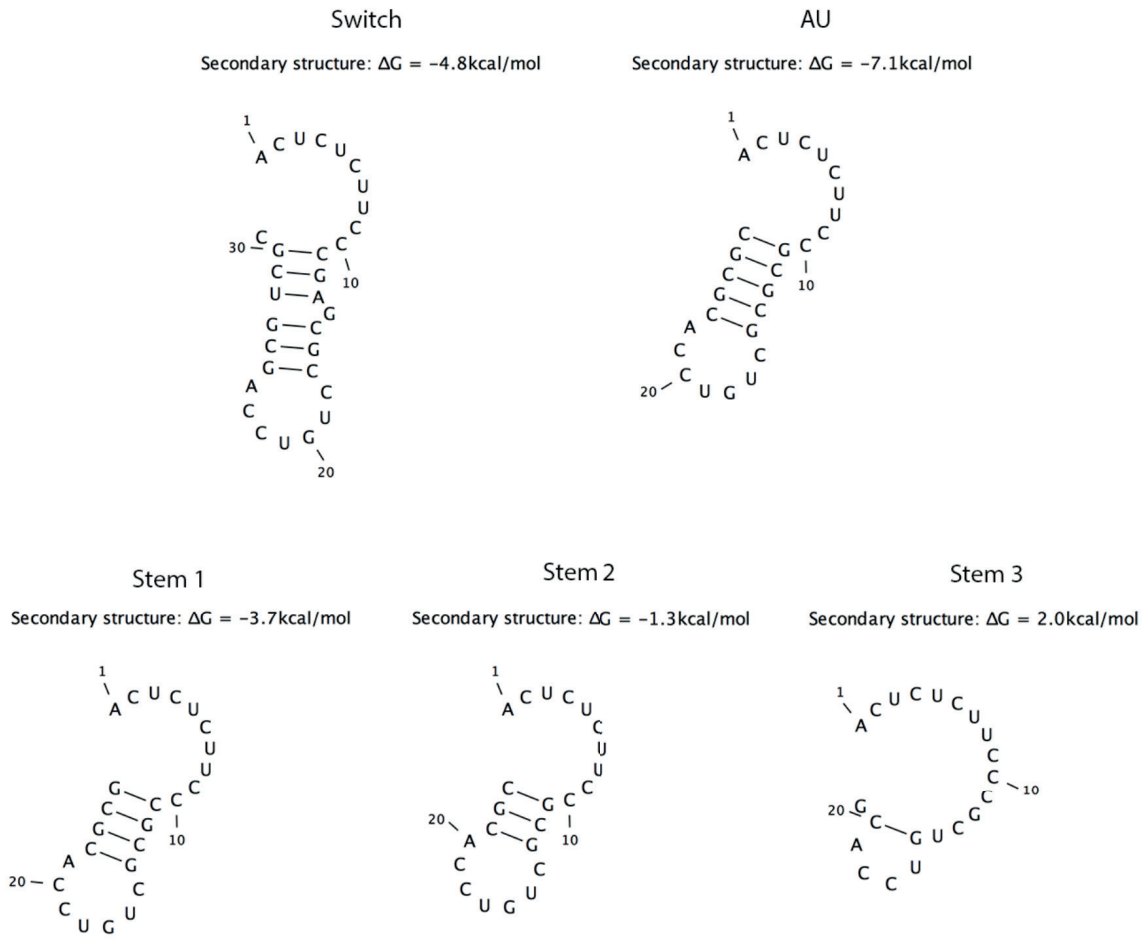

Figure 4. Primary and predicted secondary structure of the switch, AU, stem 1, stem 2 and stem 3.

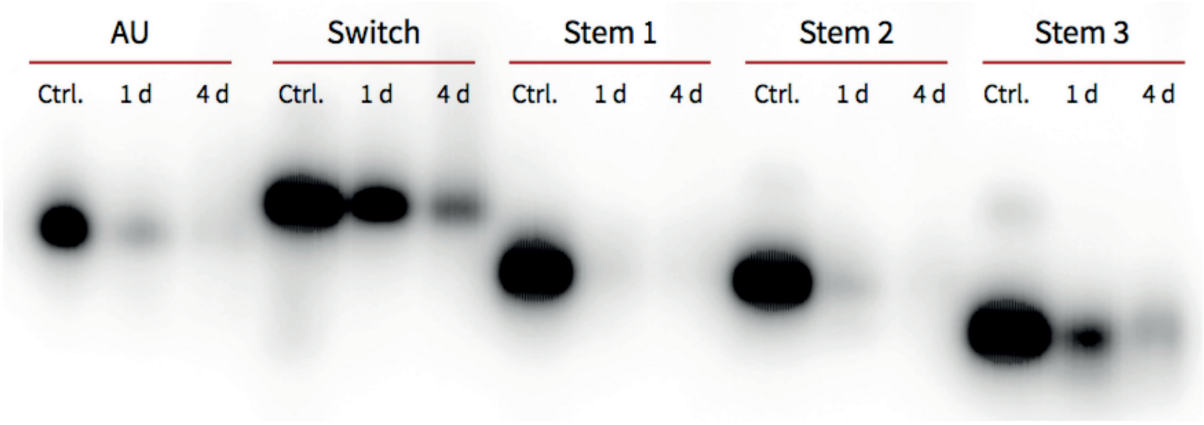

Figure 5. Stability of AU, switch, stem 1, stem 2 and stem 3 at 1 and 4 days post transfection.

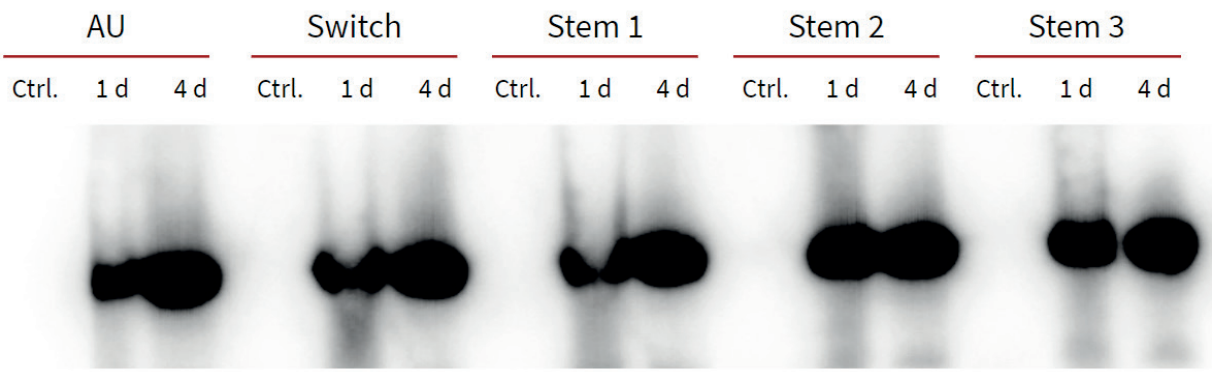

Figure 6. tRNA loading control for figure 5.

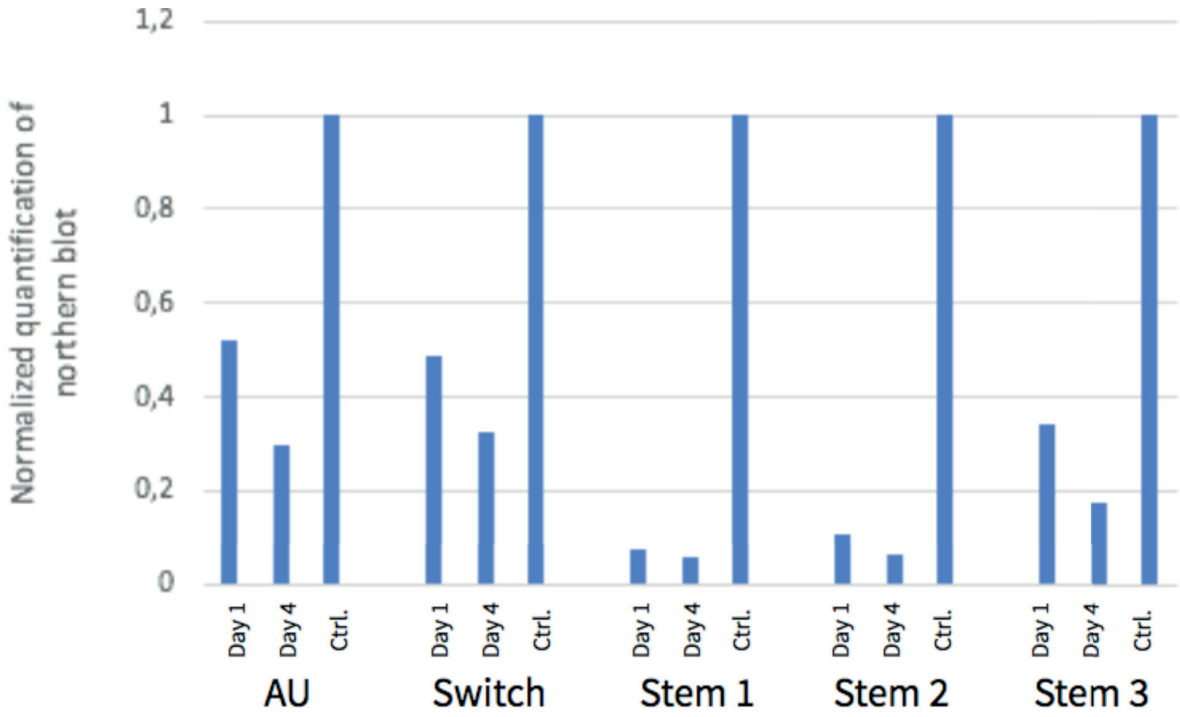

Figure 7. Normalized quantification of the data from figure 4.

To further study the stability elements additional constructs were tested. The RNAs chosen for this study had the hairpin structure mirrored (Figure 4; switch), had a deletion of mismatches in the hairpin structure (Figure 4; AU), and were successively shorter (Figure 4; stem 1-3).The results from this second study (Figure 5 and 7) show that the AU and the switch show the same rate of degradation. Stem 3 show a similar rate of degradation, but from a lower starting point. Stem 1 and stem 2 were highly unstable.

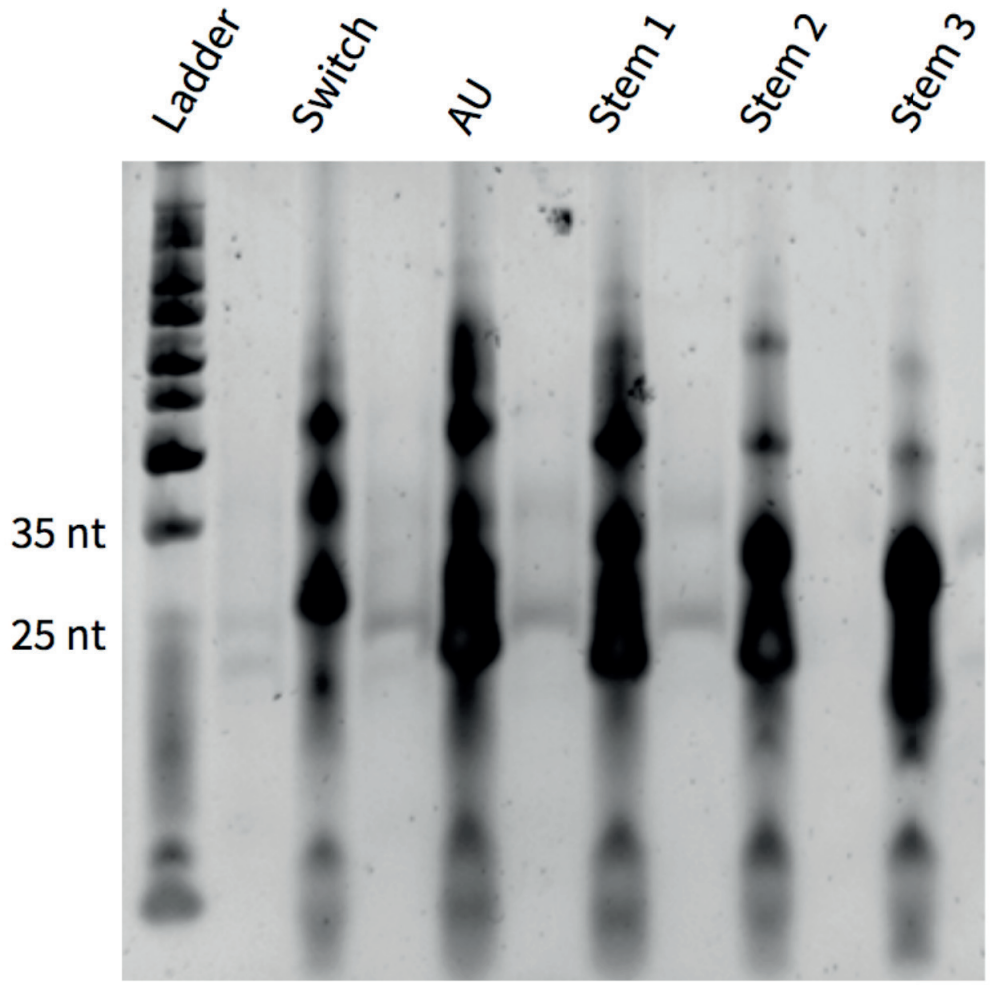

Figure 8. Validation of transcription of the RNA in figure 4.

To be able to transfect the cells with RNA, the RNA was produced by in vitro T7 RNA transcription. The result from the transcription (Figure 8) show transcription products around size 30 nt long.

The function of the sRNA is affected by the sequence of the non-base paired 5' half

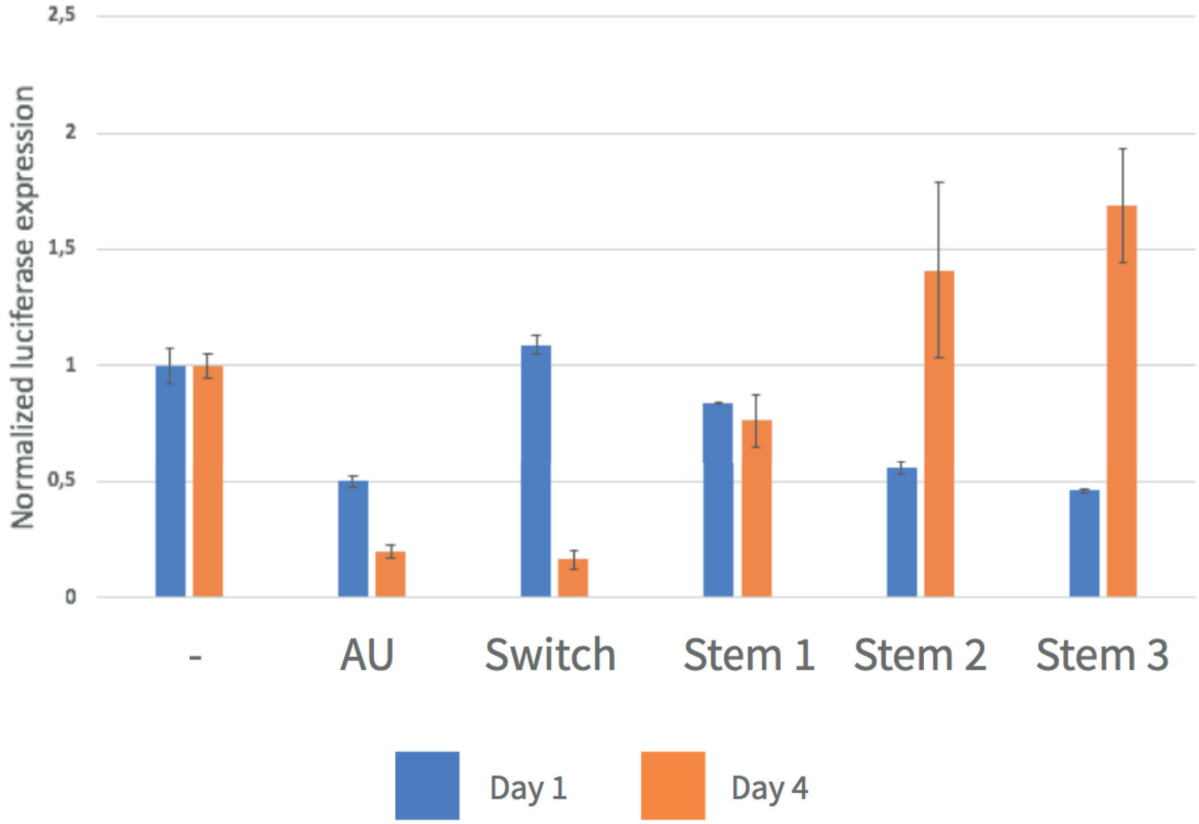

Figure 9. Normalized luciferase expression of RNA from figure 5 in a luciferase assay at 1 and 4 days post transfection of RNA.

Previous unpublished studies have shown that the function of the sRNA can be modified by changing the sequence of the non-base paired 5' half. The stability and function of the sRNAs was examined in a luciferase assay. A firefly luciferase was constructed to contain a sequence complementary to the 5' half of the sRNA. The results from this study (Figure 9) show that both AU and switch are stable at both day 1 and day 4. The stem variants show stability at day 1, but not at day 4. The results also show that the complementary sequence of the 5' half of the sRNA has a capability of downregulating the expression of firefly luciferase (Figure 9).

TSS sRNA variants are less immunostimulatory than corresponding dsRNA variants of the same sequence

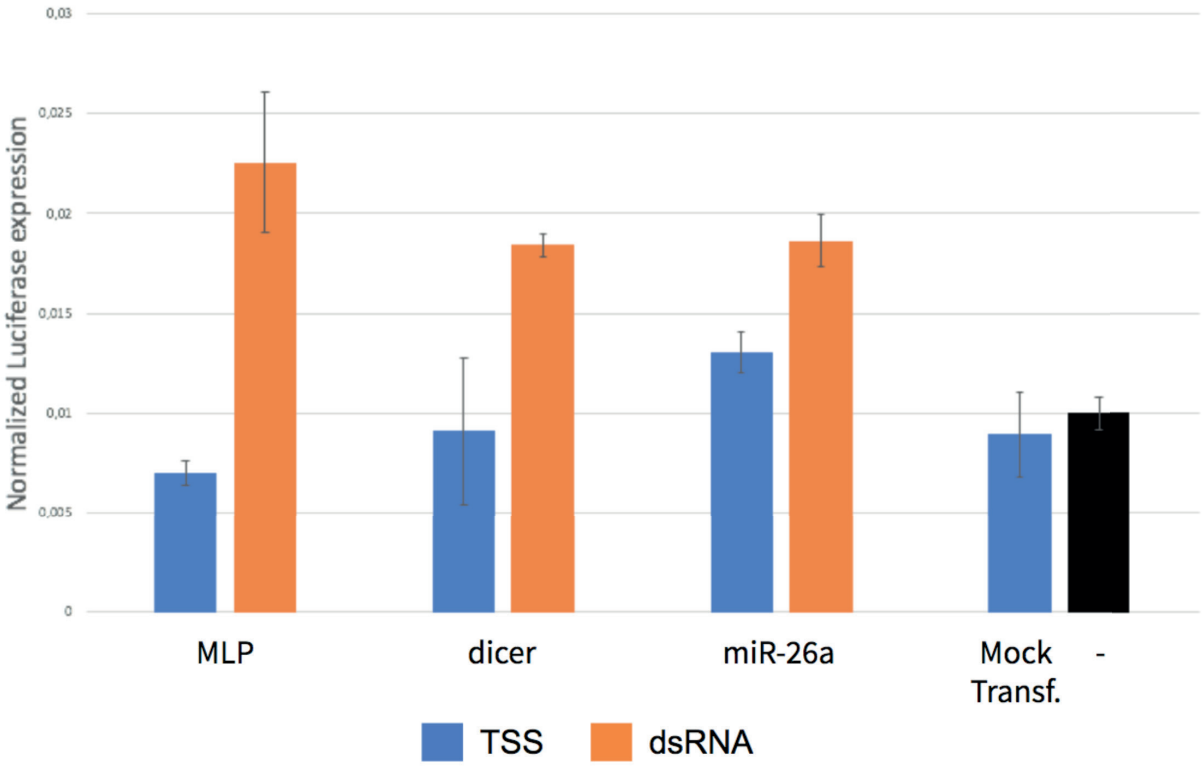

Figure 10. Normalized luciferase expression in cells transfected with NF-κB-luciferase constructs stimulated with supernatant from cells transfected with different RNA variants.

To examine the immunogenicity of the different TSS sRNA variants compared to traditional double stranded siRNAs, a luciferase assay was set up. Cells expressing a NF-κB-luciferase construct was stimulated by adding the supernatant from cells transfected with the TSS-sRNA variants and the corresponding double stranded miRNA mimics. Results from this study show that in all tested cases, the TSS-sRNA variants show less immunogenicity compared to the corresponding double stranded miRNA mimics (Figure 10).

3. Discussion

The aim of this project was to answer three aims. To assess how changing the hairpin aspects the stability of the sRNA and how the MLP-TSS-sRNA as a single stranded small RNA is protected from rapid RNA degradation in vivo, to assess how changing the non-hairpin structure affects the function of the sRNA and to assess the immunogenicity of the sRNA by measuring the amount of NF-κβ produced by cells transfected with the different RNA species.

In this project, it has been shown through the use of techniques such as northern blot and luciferase assay that the stability of the sRNA previously found (2) is dependent on its structure. By mutating the wild type MLP-TSS-sRNA in several different ways and comparing their relative stability to the wild type MLP-TSS-sRNA, the sRNA variants were found to have different rates of degradation.

Since some of the variants are similarly stable to the wild type MLP-TSS-sRNA, it can be deduced that the stability of all the variants is not dependent of a stabilizing agent that binds to the sRNA and stabilizes it. This indicates that many different modifications could be tolerated regarding the in vivo or in vitro stability of the sRNA. New modifications however need further examination as to avoid sequence dependent degradation.

It has also been shown that the structure of the non-hairpin structure of the sRNA can regulate the expression of a firefly luciferase construct containing a sequence complementary to the non hairpin structure sequence. This indicates that the non hairpin structure sequence is responsible for determining the function of the sRNA. This is in line with the results from previous studies done on the subject where sRNA targeting different proteins were shown to downregulate the specific proteins, without a general downregulation of all proteins (unpublished).

Thirdly it was shown that the sRNA show less cytokine production than their dsRNA counterparts. Some sRNA molecules show less cytokine response than others, meaning that there could be some sequence specific effect in the activation of the cytokine production. This indicates that the immunologic response is lower and that the sRNA could work therapeutically without major complications. Specific sRNA molecules however need to be further examined as to avoid sequence specific activation of the immune system.

DISCUSSION

Looking at the results regarding the stability of the sRNAs and comparing it to their predicted stability it looks as if the predicted stability is accurate at predicting the stability. It however looks as samples stem 1 and stem 2 did not work properly. They may need to be repeated, to confirm if their expression is as low as the results show or if it is some methodological error behind the result.

It is also rather odd that the samples stem 2 and stem 3 show an upregulation at day 4 in the luciferase structural assay compared to the mock transfection. This may need further investigation.

What this project has shown ist that this new sRNA could play a vital part of the future of biologically based therapeutics, alongside the use of for example antibodies. By modifying the sequence of the sRNA it is possible to alter its characteristics with regards to stability, specificity and immunogenicity. In some applications the half life need to be short, which requires a specific stability. In some other applications, for example in some types of cancers, the immune response need to be initiated giving rise to a specific immunogenicity of the sRNA.

Apart from the therapeutic value, the sRNA could also play a role in regulating gene expression in vitro as a complement to gene silencing techniques such as crispr-cas and other types of knockdown techniques.

4. Methods and material

Cell culture

HeLa-cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 1% penicillin/streptomycin (PEST) at 37°C in 7% CO2.

Transfection and transcription

Transfection of siRNA was done using the Jetprime transfection reagent (Polyplus-transfection) according to the instructions supplied by the manufacturer.

Transcription of siRNA was done using the MEGAshortscript T7 (Invitrogen) according to the instructions supplied by the manufacturer.

RNA extraction

RNA extraction was done using TRI reagent (Sigma). The cells were washed with 1xPBS. 500 µl of TRI reagent was added to each of the wells. The plates were stored in -80°C until use. The plates were thawed and the contents of the wells were transferred to eppendorf tubes containing 100 µl chloroform. The samples were vortexed for 15 s, followed by incubation at RT for 10 min. The samples were spun down at 11.2 kRPM, 4°C for 10 min. The aqueous phase was transferred to new eppendorf tubes containing 600 µl 99.5% ethanol, 30 µl 3 M sodium acetate and 1 µl glycogen. The samples were vortexed for 10s and left incubating in RT for 5 min.

RNA extraction was also performed using buffer3 complemented by DNAse. The cells were washed once with 1xPBS followed by adding 300 µl of buffer3 supplemented with DNAse I. The plate was shaken on ice in 4°C for one hour. The lysate was transferred to eppendorf tubes, which were then sonicated for 40 cycles. 300 µl phenol/chloroform/IAA was added to each sample. The samples were vortexed for 10 minutes, followed by centrifugation at 13 kRPM, RT for 5 min. The supernatant was transferred to new tubes containing 900 µl 99.9% ethanol, 30 µl 3M sodium acetate and 1 µl glycogen. The samples were vortexed for 1 minute and stored in -20°C until use.

Northern blot

Northern blot was performed on extracted RNA. The samples were spun down at 11.2 kRPM, 4°C for 8 min. The supernatant from the samples was removed and the pellet was resuspended in 500 µl 75% ethanol. The samples were spun at 15 kRPM, 4°C for 5 min and the supernatant was removed. The pellets were dissolved in 10 µl loading dye.

14% denaturing polyacrylamide gels were cast for running the RNA by mixing 5 ml prepared 14% acrylamide mix, 20 µl TEMED and 50 µl APS and casting it between glass sheets.

The samples were loaded and run at 100 V until finished. The gel was placed on top of three whatman papers in the membrane transfer machine along a nylon membrane and three more whatman papers. The lid was closed and the transfer was run for 30 minutes at 20 V. The membrane was then placed on top of a EDC-solution soaked whatman paper on a glass sheet. The membrane, paper and plate was wrapped and placed in 50°C for 1 hour. The membrane was rinsed in dH2O and placed in a cylindrical glass flask along 4 ml of ULTRA hybridization bufferin a rotating incubator at 42°C for 4 hours.

RNA probe was prepared by mixing 30.5 µl dH2O, 5 µl 10 pmole/µl oligonucleotide, 5 µl T4 PNK buffer, 2 µl T4 PNK enzyme and 7.5 µl γ-ATP. This was incubated at 37°C for 1 hour. A sephades spin column was prepared by resuspending the solution in the spin column by vortexing. The column was placed in a collecting tube and spun down at 2.7 kRPM for 1 minute. The column was transferred to an eppendorf tube. The probe solution was added to the column, which was again spun down at 2.7 kRPM for 1 minute. The column was discarded and the spun out solution was added to the ULTRA hybridization buffer in the cylindrical glass flask. The flask was then placed back in the rotating incubator overnight.

The membrane was then washed 3 times for 45 minutes with wash buffer. The membrane was then placed in plastic on top of a whatman paper in a scanning cassette until sufficient exposure.

Luciferase assay

The luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase constructs with and without

binding sites for the MLP-TSS-sRNA was used for one part of the project and NF-κβ reporter luciferase constructs were used for the toxicity study. Nano-luc was used as a normalising control for all luciferase assays. The luciferase activity was measured using the Infinite M200 luminometer (Tecan). Results shown are the normalized ratios of the luciferase constructs to nano-luc from three replicates.

5. References

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METHODOLOGICAL CONSIDERATIONS

Annex 1: Methodological considerations

Northern blot is a method for separating RNA depending on size and then being able to quantify the amount of RNA existing in the sample. This method has been used for a long time, and has its advantages and its disadvantages. It is good for running small RNA, compared to using for example a qPCR. It however takes time. In this particular project one of the studies did not work for several northern blots, meaning that the study took more time than maybe would be necessary using another method.

Luciferase assay is another way of measuring the expression of different proteins and some RNA. It is a quick method for getting data, compared to for example a northern blot, but it does not have the same capabilities as to getting quantifiable data.

ETHICAL CONSIDERATIONS

Annex 2: Ethical considerations

No animals or people were used in this study. If successful, this study could help in the development of new siRNA and miRNA based therapeutics used to treat difficult to treat diseases. Today, the use of siRNA and miRNA for treatment is associated with problems regarding the efficacy, the immunogenicity and the specificity. The use of shorter and more efficient sRNA variants could be a way of solving these problems and giving treatment to more patients.

POPULAR SCIENTIFIC SUMMARY

Annex 3: Popular scientific summary

Många människor lever idag med komplicerade genetiska sjukdomar som inte har någon bra behandling. Svårbehandlad cancer, utdragen diabetes och kronisk inflammation är bara några av alla sjukdomar som behöver bättre behandlingsmetoder.

Nyligen har forskare hittat en ny molekyl som kan användas som behandlingsmetod för just komplicerade genetiska sjukdomar. Denna molekyl kallas sRNA och den liknar det genetiska material kroppen själv har för att göra de byggstenar kroppen behöver. Denna fungerar genom att ett enzym i kroppens celler använder detta sRNA för att hitta den gen som behöver nedregleras. Detta sRNA passar bara på en gen, likt en pusselbit.

Forskarna undersökte hur sRNA:t kan modifieras för att ändra dess egenskaper, exempelvis stabiliteten eller funktionen på sRNA:t. De såg då att det är möjligt att göra ganska stora modifieringar utan att det förändrade hur sRNA:t fungerade. Genom att göra sRNA:t mindre blev det mindre stabilt och genom att ändra sRNA:t på andra sätt går det att reglera andra gener.

Detta innebär att det kan vara möjligt att använda detta sRNA som en framtida behandling för vissa sjukdomar som idag är svåra att behandla. Det kommer fortfarande i många fall behövas livslånga behandlingar, men förhoppningsvis med mindre biverkningar och med bättre effekt.