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Synthesis and *in vitro* Assessment of Chemically Modified siRNAs Targeting *BCL2* that Contain 2'-Ribose and Triazole-linked Backbone Modifications

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Gordon Hagen,[a] Brandon J. Peel,[a] John Samis[b] and Jean-Paul Desaulniers[a]

Short-interfering RNAs (siRNAs) are naturally occurring biomolecules used for post-transcriptional gene regulation, and therefore hold promise as a future therapeutic by silencing gene expression of overexpressed deleterious genes. However, there are many inherent problems with the native RNA structure. This project investigates the ability of a library of chemically modified siRNAs to target the therapeutically relevant oncogene, *BCL2*, by combining 2'-ribose sugar modifications with a novel triazole-linked backbone modification, previously described by our group. Solid support phosphoramidite chemistry was used to incorporate chemical modifications at various positions within anti-*BCL2* siRNAs. *In vitro* effects were evaluated through qPCR, cell viability, nuclease stability, and an ELISA assay. Our results indicate that these unique modifications are well tolerated within RNAi and show enhanced activity and stability over natural siRNAs, while also improving upon inherent issues of toxicity and immunological activity.

Short-interfering RNAs (siRNAs) are a class of biomolecules characterized by their ability to silence gene expression at the translational level through the RNA interference (RNAi) pathway.[1,2] RNAi is an innate cellular pathway that involves short double-stranded RNA molecules bound to the RNA-induced-silencing-complex (RISC). RISC unwinds and dissociates the sense RNA strand and retains the antisense RNA strand to create an 'active-RISC-RNA-complex'. This active complex binds to a complementary mRNA transcript in a sequence-dependent manner, and through the action of Argonaute 2, catalytically degrades the mRNA.[3] siRNAs hold promise as a future therapeutic, as many diseases are characterized by aberrant gene expression.[4,5] SiRNAs are widely used as a research tool, probing molecular function by suppressing gene expression, but have yet to be utilized as a therapeutic due to several limitations. One major limitation of siRNAs is the susceptibility to degradation by endogenous nucleases, which causes siRNAs to have a very short half-life and decreased potency *in vivo*.[6] Another major limitation of siRNA therapeutics is the propensity to illicit an innate immune response causing pro-inflammatory cytokines to be released, leading to a systemic immune response.[7] SiRNAs are also prone to cause off-target effects, leading to suppression of non-target mRNA sequences.[8] Finally, siRNAs have difficulty being internalized by cells, thus making their bioavailability a challenge. These limitations are often due to the native structure of RNA which is recognized by endogenous proteins. However, through the use of chemical modifications, many of these pharmacological shortcomings and limitations can be modulated.[9]

Some of the most common chemical modifications used in oligonucleotide design are 2'-ribose modifications. These modifications involve replacing the natural 2'-hydroxyl group with another functional group, such as a 2'-*O*-methyl (2'-*O*-Me) and/or 2'-fluoro (2'-F).[10] 2'-ribose modifications are becoming more common in oligonucleotide design, and their building block phosphoramidites are commercially available for solid-phase oligonucleotide synthesis.[10] When these modifications are used within siRNA macromolecules in biological experiments, data has demonstrated an increase in the nuclease stability of RNA duplexes,[11] a decrease in the immune response,[12] and an increase in the thermodynamic stability of the siRNA duplex.[13] Backbone modifications, which replace the native phosphodiester linkage, are another less common chemical modification employed in oligonucleotide synthesis to overcome therapeutic barriers.[14] One such example is the non-ionic triazole-linked nucleic acid dimer, previously described by our research group.[15-17] When this backbone modification was incorporated into an siRNA, gene-silencing data illustrated dose-dependent knockdown of an exogenous

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gene *in vitro* and improved serum nuclease resistance when compared to an unmodified siRNA.[16] Oligonucleotide therapeutics which are commercially available contain multiple types of chemical modifications within their structure, but currently no universally acceptable chemical modification design exists for siRNAs, despite some significant advances in the field.[18]

The triazole-linked backbone modification developed by our laboratory has yet to be investigated for immune stimulation or for its use in gene-silencing clinically relevant endogenous genes. The gene in this study that we examine is *BCL2. BCL2* encodes the bcl2 protein, which is an important regulator of type I and II programmed cell death (apoptosis and autophagy, respectively).[19-21] This gene can become mutated into an oncogene through a translocation event which causes overexpression of *BCL2,* thus leading to decreased cell death and increased cellular proliferation.[19-21] *BCL2* is associated with several clinical cancer types and is implicated in chemotherapeutic resistance, thus making *BCL2* a good therapeutic target for siRNA design.[22]

This paper aims to generate chemically-modified siRNAs that target the clinically relevant oncogene, *BCL2, in vitro.* These siRNAs contain a uracil-triazole-uracil backbone modification in conjunction with 2'-ribose modifications to determine if therapeutic limitations of nuclease susceptibility and immune activation can be modulated in an attempt to further siRNA therapeutic design.

A library of anti-*BCL2* siRNAs were generated using solid support phosphoramidite chemistry (see Table S1 in the Supporting Information). The library of siRNAs contains a combination of 2'-ribose modifications in conjunction with a uracil-triazole-uracil backbone (**U$_t$U**) modification (Fig. 1). All chemically-modified siRNAs generated retain standard A-type helical conformation, as confirmed by circular dichroism (see Fig. S4 in the Supporting Information). Both negative and positive circular dichroism bands at approximately 210 nm and 265 nm, respectively, are observed.

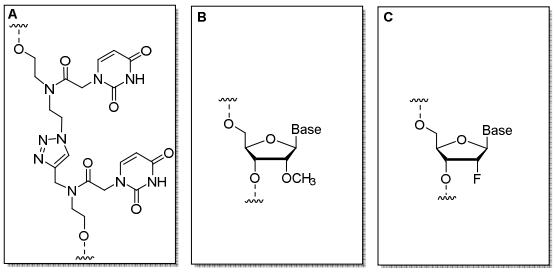

Fig. 1. Structures of chemical modifications used in the synthesis of anti-*BCL2* siRNAs. A) uracil-triazole-uracil dimer (**U$_t$U**). B) ribose 2'-*O*-methyl (2'-*O*-Me) nucleoside; C) ribose 2'-fluoro (2'-F) nucleoside.

We have previously described optimal placement of **U$_t$U** modifications within siRNAs,[16] thus **U$_t$U** modifications were placed at either the 3'-overhang position or within the 3'-region of the sense strand. The 2'-ribose modifications were placed in siRNA duplexes either with or without **U$_t$U** modifications. The 2'-ribose modifications were placed at various positions on the sense strand; they were placed around the 5'-end region or throughout the entirety of the strand. Alternatively, the 2'-ribose modifications were placed on the pyrimidyl-linked ribose sugars throughout the antisense strand. The ability of each siRNA to knockdown *BCL2* mRNA was assessed at three concentrations (20 nM, 10 nM and 1 nM), 24 hours post-transfection in KB cells by measuring normalized expression levels using qPCR. Gene-silencing data from the chemically modified siRNAs show effective dose-dependent down-regulation of *BCL2* at all concentrations (see Fig. S1 in the Supporting Information). Several of the chemically-modified siRNAs exhibit enhanced activity compared to unmodified wild-type siRNA (**Bwt**) at concentrations of 10 nM and below. From the initial screen of the siRNA library, eight siRNAs were chosen for further investigation because of enhanced dose-dependent knockdown of *BCL2* compared to **Bwt** (Table 1).

Table 1. Sequences of selected anti-*BCL2* siRNAs.

RNA	siRNA duplex
Bwt	5'-GCCUUCUUUGAGUUCGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B2	5'-GCCUUCUUUGAG**U$_t$U**CGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B3	5'-GCCUUCUUUGAGUUCGGUG**U$_t$U**-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B4	5'-G**CCUUCUUU**GAGUUCGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B5	5'-G**CCUUCUUU**GAG**U$_t$U**CGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B6	5'-G**CCUUCUUU**GAGUUCGGUG**U$_t$U**-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B10	5'-G**CCUUCUUU**GAGUUCGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B11	5'-G**CCUUCUUU**GAG**U$_t$U**CGGUGtt-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
B12	5'-G**CCUUCUUU**GAGUUCGGUG**U$_t$U**-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
NC	5'-CUUACGCUGAGUACUUCGAtt-3' 3'-ttGAAUGCGACUCAUGAAGCU-5'

U$_t$U corresponds to the uracil-triazole-uracil modification; **C** or **U** corresponds to the 2'-*O*-Me modification; **C** or **U** corresponds to the 2'-F modification. **NC** is a negative control siRNA and corresponds to a sequence which does not target endogenous mRNA. The top strand corresponds to the sense strand; the bottom strand corresponds to the antisense strand. In all duplexes, the 5'-end of the bottom antisense strand contains a 5'-phosphate group.

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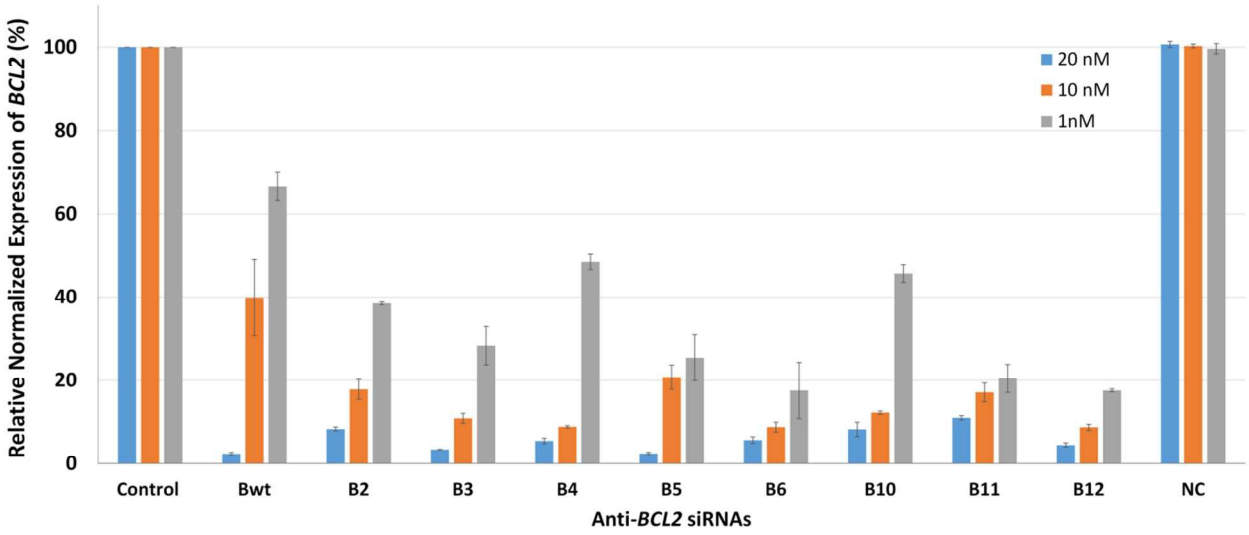

Fig. 2. Relative gene expression of *BCL2* in KB cells 24 hours post anti-*BCL2* siRNA transfections at 1nM, 10nM and 20nM. Gene expression was measured using qPCR and expression was normalized to an untreated control using reference genes 18s rRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

When **U$_t$U** modifications were combined with 2'-ribose modifications clustered around the 5'-end of the sense strand, gene silencing ability was enhanced compared to **Bwt** at concentrations above 1 nM (Fig. 2). When the **U$_t$U** modification was placed in an overhang position (**B3**, **B6**, and **B12**), whether in conjunction with 2'-ribose modifications or not, the siRNAs showed the greatest silencing ability at all concentrations while retaining a dose-dependent response. This data indicates that the chemical modification patterns chosen for this study are amendable within the RNAi pathway, and have excellent gene silencing ability.

After mRNA knockdown was assessed, it was relevant to investigate the biological stability of these siRNAs in the presence of nuclease enzymes. Independently, it has been previously reported that both **U$_t$U** and 2'-ribose modifications incur nuclease stability when compared to unmodified siRNAs.[11,16] Unmodified and modified siRNAs were incubated in fetal bovine serum (FBS) for up to five hours to analyze the truncated products in a time-dependent manner using polyacrylamide gel electrophoresis (Fig. 3).

Unmodified siRNA (**Bwt**) degraded almost immediately, with little to no siRNA present within 0.5 hours of incubation in the presence of nucleases. When the **U$_t$U** modification was incorporated internally within the siRNA (**B2**), the full siRNA duplex persisted for up to 2 hours, and when placed at an overhang position (**B3**), the duplex was persistent for up to 4 hours. 2'-*O*-Me modifications clustered at the 5'-region of the sense strand (**B4**) increased nuclease stability, allowing the siRNA duplex to last for 2 hours in the presence of nucleases. When the 2'-*O*-Me modification was combined with an internal **U$_t$U** modification (**B5**), the duplex lasted up to 3 hours. When the 2'-*O*-Me modification was combined with the **U$_t$U** overhang (**B6**), the siRNA duplex was still present after 5 hours of incubation in the presence of nucleases. When the 2'-*O*-Me modification was combined with the **U$_t$U** backbone modification, nuclease resistance was greatly increased. Any

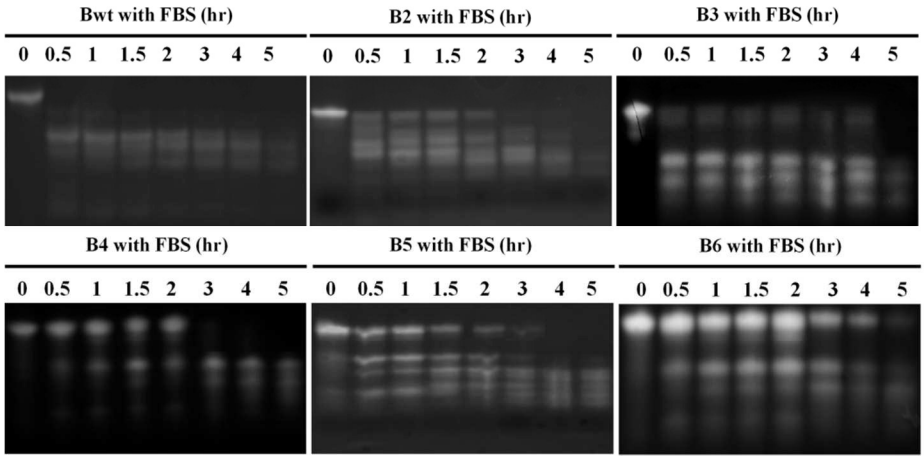

Fig.3. Nuclease stability assay. 20% Non-denaturing polyacrylamide gel with degradation products of siRNAs **Bwt** and **B2-B6** after incubation with 13.5% fetal bovine serum at 37.5 °C from 0 hours to 5 hours.

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change to the native chemical structure caused a significant increase in nuclease stability, especially when backbone and ribose modifications were combined.

In vivo, double-stranded RNAs stimulate innate immunity by triggering the type I interferon response.[7] *In vitro*, the immune response is highly dependent on the specific cell line used, and many cell lines do not possess the receptors necessary to respond to siRNAs, whereas using human peripheral mononuclear cells (PBMC) isolated from whole human blood serves as a fast and reliable *in vitro* approach to predict *in vivo* response.[23] Therefore, after obtaining ethical approval, human PBMCs were isolated from whole blood donated by healthy volunteers after obtaining written consent. PBMCs were stimulated using 20 nM of anti-*BCL2* siRNAs complexed with lipofectamine 2000. After 24 hours, human interferon alpha (IFN-α) levels were measured using a VeriKine ELISA kit (PBL Assay Science) according to the manufacturer's protocol (Fig. 4).

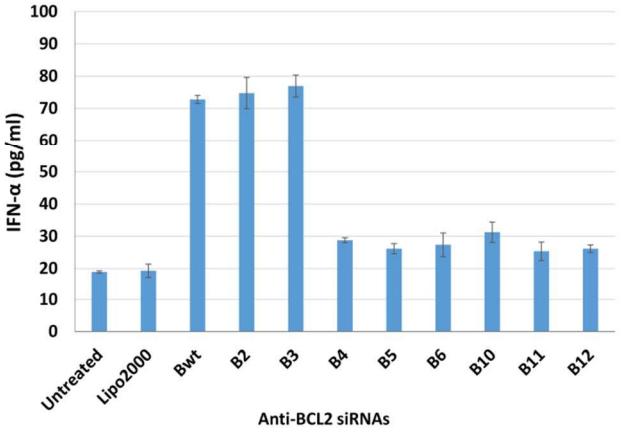

Fig. 4. Human Interferon Alpha production in response to anti-*BCL2* siRNAs. PBMCs were stimulated with 20 nM of anti-*BCL2* siRNAs supplemented with Lipofectamine 2000. IFN-α was measured in the supernatant 24 hours post-transfection by ELISA.

PBMCs challenged with unmodified siRNA as well as triazole modified siRNAs (**Bwt**, **B2**, and **B3**) induced IFN-α production (72.8, 74.4, and 76.9 pg/ml respectively), whereas siRNAs containing 2'-ribose modifications (**B4**, **B5**, **B6**, **B10**, **B11** and **B12**) showed a decrease in immune response similar to untreated controls (28.8, 26.1, 27.3, 31.2, 25.4, and 26.1 pg/ml respectively). This data suggests that the **U$_t$U** modification alone does not alter immune response in comparison to unmodified siRNA (**Bwt**). However, when the **U$_t$U** modification was combined with 2'-ribose modifications the innate immune response was diminished as indicated by a decrease in IFN-α production from PBMCs.

The siRNAs in this study target a clinically relevant oncogene, *BCL2*. *BCL2* oncogene is responsible for the overproduction of the bcl2 protein, which is a key regulatory protein in cellular apoptosis. Bcl2 prevents pro-apoptotic factors from triggering type I and type II cell death, and thus overexpression of *BCL2* prevents cells from undergoing apoptosis.[19, 20, 21] KB cells are known to overexpress the *BCL2* oncogene. Therefore, decreasing *BCL2* expression, via RNAi should cause a decrease in cellular proliferation. An XTT cellular proliferation kit (ATCC) was used to determine proliferation rates of KB cells treated with anti-*BCL2* siRNA in comparison to untreated cells 24 hours post siRNA transfection (see Fig. S3 in the Supporting Information). The anti-*BCL2* siRNAs were able to decrease cellular proliferation by 20% or more at all concentrations when compared to untreated cells. To ensure that the decreased cellular proliferation was not due to toxicity from the siRNAs, a negative control was used, which did not target an endogenous gene; the negative control exhibited the same cellular proliferation rate as the untreated control. From the qPCR screen, the best candidate siRNAs (Table 1) were used to determine cellular proliferation rates from continual knockdown over the course of four days (see Fig. S2 in the Supporting Information). All of the best candidate siRNAs were able to decrease cellular proliferation within 24 hours by at least 30% when compared to untreated controls (Fig. 5).

Cellular proliferation continued to decrease after 48 and 72 hours. By 96 hours, cellular proliferation rates stabilized at their lowest rate when compared to the untreated control. Continual knock-down of *BCL2* caused a 50-60% decrease in cellular proliferation after 96 hours compared to the untreated control.

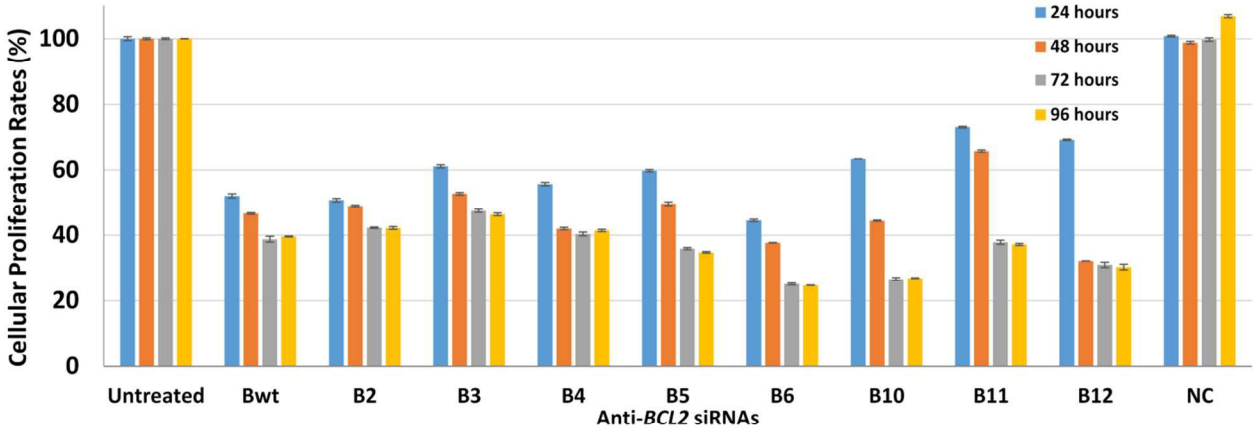

Fig. 5. Time course cellular proliferation assay. Relative cellular proliferation rates of KB cells treated with 20 nM of anti-*BCL2* siRNAs compared to untreated KB cells. Cellular proliferation rates were measured using XTT reagent at 24, 48, 72 and 96 hours.

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Conclusions

siRNA oligonucleotides containing commercially available 2'-ribose modifications (2'-*O*-Me/2'-F) can be combined with a novel triazole-linked backbone modification in siRNA design and these siRNAs are amendable within the RNAi pathway. The qPCR *BCL2* screens show that these modifications have enhanced gene-silencing activity either independently or in conjunction within the same siRNA. As expected, the chemically modified siRNAs also show excellent biological stability with high nuclease resistance, whereas unmodified siRNAs are readily degraded by serum nucleases.[6,11,16] Nuclease stability was additive when combining triazole-linked backbone modifications with 2'-ribose modifications, especially when the triazole modification was placed at the overhang position. This enhanced biological stability is desirable for pharmaceutical applications.

Another major limitation of the pharmaceutical application of siRNAs is the innate immune response. The innate immune system has a variety of receptors which recognize and respond to small nucleic acids, triggering cytokine and interferon release.[7] Both 2'-*O*-Me and 2'-F modifications block immune response *in vitro* and *in vivo*,[24] whereas the triazole-linked nucleic acid has not been tested for immune response. Our results show that triazole-linked backbone modifications illicit a similar immune response as unmodified siRNAs, and are detected by the innate immune system. When the triazole-linked backbone modification is combined with 2'-ribose modified siRNAs, the immune response is diminished to a similar level as induced by siRNAs containing only 2'-ribose modifications. Overall, this shows that the triazole-linked nucleic acid can be combined with commercially available 2'-ribose modifications to evade immune response while still maintaining RNAi efficacy.

Furthermore, the utility of these siRNAs were assessed by targeting a clinically relevant oncogene, *BCL2*, which is implicated in a diverse range of cancers.[22] Not only were the siRNAs successful in knocking down *BCL2* expression *in vitro*, but were also able to decrease cellular proliferation rates. These results mirror those of oblimersen sodium (Genasense), a *BCL2* antisense oligonucleotide. In preclinical trials, Genasense depleted *BCL2* mRNA which correlated to increased apoptosis and decreased cellular proliferation *in vitro*. [27, 28]

Overall, by combining 2'-ribose modifications with a novel triazole-linked backbone modification, we have generated siRNAs with chemical modification patterns which have enhanced nuclease stability and decreased immune activity without compromising the potency of the duplex as a trigger of RNAi.

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Notes and references

[a]Faculty of Science, University of Ontario Institute of Technology. Oshawa. ON. L1H 7K4. Jean-Paul.Desaulniers@uoit.ca
[b]Faculty of Health Science, University of Ontario Institute of Technology. Oshawa. ON. L1H 7K4.

Electronic Supplementary Information (ESI) available: [Full list of siRNAs synthesised and assessed as well as detailed experimental materials and methods can be found in the Electronic Supplementary Information]. See DOI: 10.1039/c000000x/

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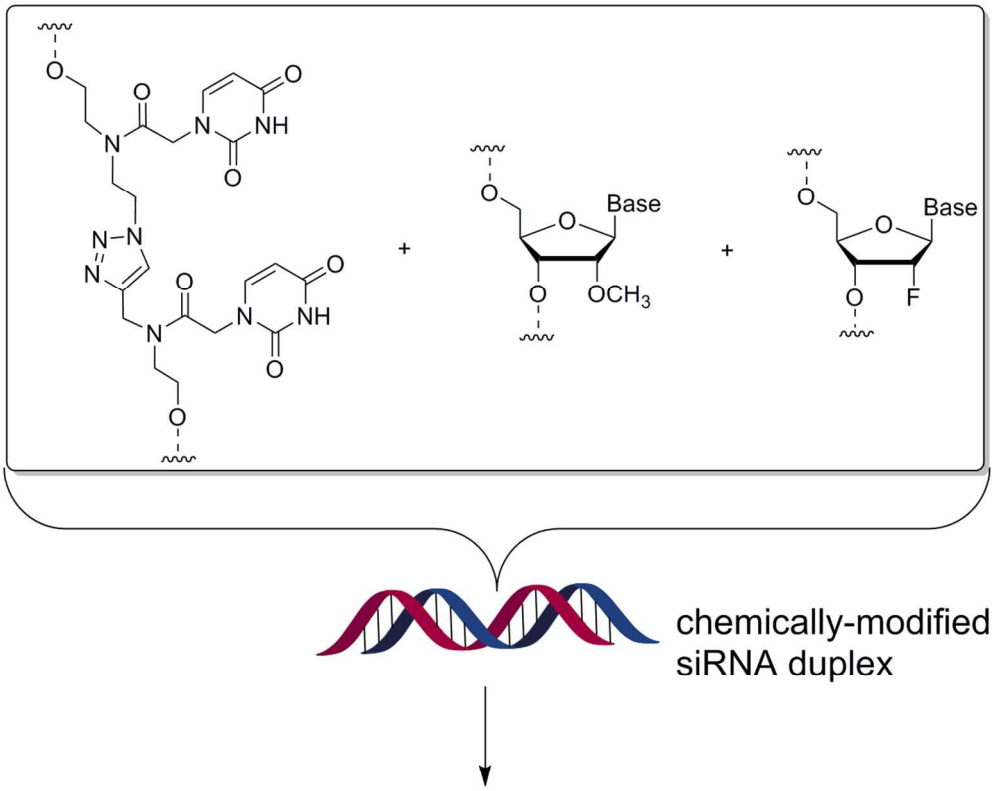

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