ACS Medicinal Chemistry Letters

Letter

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Conjugation and Evaluation of Small Hydrophobic Molecules to Triazole-Linked siRNAs

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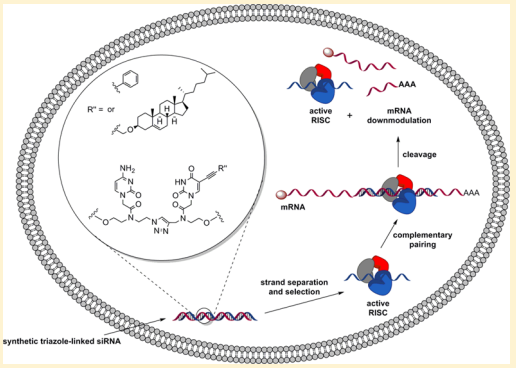
Supporting Information

ABSTRACT: Short interfering RNAs (siRNAs) have tremendous potential as a new class of next-generation therapeutics; however, their progress is lagging due to issues related to stability, biodistribution, and cell-membrane permeability. To overcome these issues, there is widespread interest in chemically modifying siRNAs. In this study, siRNAs that contain a triazole-backbone unit with pyrimidine-modified hydrophobic substituents were synthesized and examined for their gene-silencing activity. In our study, we generated a library of siRNAs that target both a plasmid reporter system and an endogenous gene target, bcl-2. Our results indicate that these unique modifications are well tolerated within the RNA interference pathway. In addition, a cholesterol-modified triazole-linked siRNA targeting the exogenous target *firefly* luciferase was capable of gene-silencing at levels greater than 80% in the absence of a carrier complex.



KEYWORDS: *Short interfering RNAs, nucleic acid chemistry, oligonucleotides, gene expression, down regulation*

RNA interference (RNAi) is a gene-silencing endogenous biological pathway that utilizes duplex siRNAs as substrates for activation.[1,2] RNAi has promise as a future therapeutic due to its effective gene-silencing profile in a variety of systems.[3,4] However, some of the challenges with double-stranded RNA as a prospective candidate for treating disease include its labile and polyanionic phosphate backbone profile.[5,6] In addition, there are significant challenges associated with cell-membrane permeability and delivery.[7−9] Some ways to overcome the limitations of the native RNA structure are to modify its inherent properties through the use of chemical modifications. Previous work from our group has shown that nonionic backbone units such as triazoles and amides function effectively within siRNAs.[10,11] Based on the prior success of triazoles as backbone replacements within siRNAs, we sought to further modify the phosphoramidite building block with hydrophobic small molecules in order to improve its overall hydrophobic character and properties.

The incorporation of various small molecules conjugated to siRNA has been explored to assist in the biodistribution and to improve the properties of the duplex RNA within the cytoplasm of the cell.[12−15] One approach involves incorporating hydrophobic groups such as cholesterol to the siRNA to improve its pharmacokinetic and pharmacodynamic properties.[13,16,17] Many different novel approaches have been reported to incorporate cholesterol within the internal region of siRNAs.[13] However, these approaches are typically prepared in the absence of backbone modifications. Methods to incorporate small molecule conjugate modifications at the 3′ and 5′ end of oligonucleotides are well established,[18,19] and several commercially available options exist. However, these strategies cannot be used to incorporate small hydrophobic molecules within the internal region of the siRNA while retaining the oligonucleotide targeting sequence. The goal of this study is to synthesize and evaluate siRNAs that contain both the desirable features of a nonionic triazole-backbone linkage and a small hydrophobic moiety.

In our strategy, we incorporated a phenyl group and a cholesteryl group at position 5 of a uracil residue using Sonogashira cross-coupling chemistry[20] to a triazole-linked molecule. Through phosphoramidite chemistry, we synthesized siRNAs bearing these modifications. We then evaluated the characteristics of these siRNAs for RNAi activity by using an *in vitro* HeLa cell line bearing plasmids encoding *firefly* luciferase and Renilla, and also targeting the endogenous target *bcl-2*.

To the best of our knowledge, this is the first time that the effect of a nonionic linkage unit derivatized with a base-modified hydrophobic group has been explored within siRNAs. Several other examples of base-modified siRNAs have been explored; however, they have been utilized within the context of a sugar linked to a natural phosphodiester backbone.[5,21,22] Herein, we report the synthesis of siRNAs bearing a nonionic linkage derivatized with base-modified hydrophobic groups (Figure 1). These siRNAs exhibit potent gene-silencing characteristics. Furthermore, some of our chemically derivatized siRNAs offer effective gene-silencing in the absence of a carrier transfection reagent.

Received: June 22, 2014
Accepted: December 4, 2014

dx.doi.org/10.1021/ml500260j | *ACS Med. Chem. Lett.* XXXX, XXX, XXX−XXX

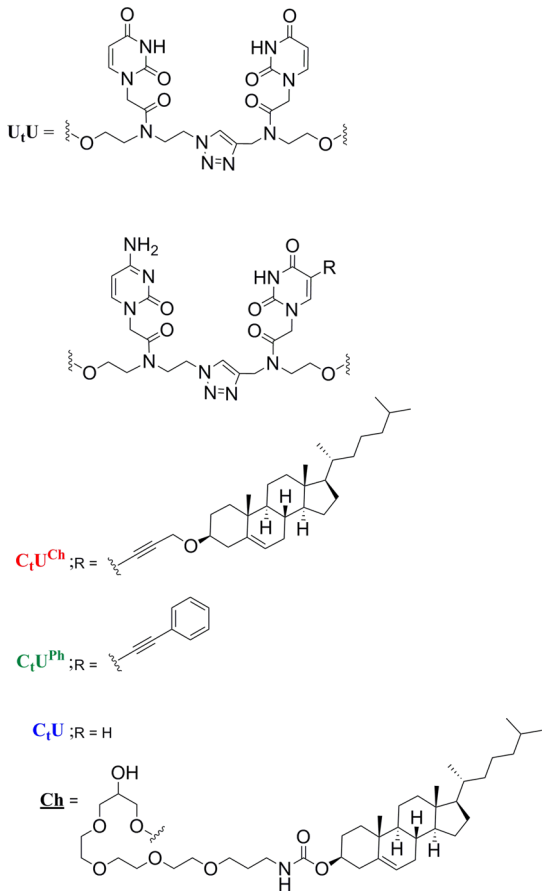
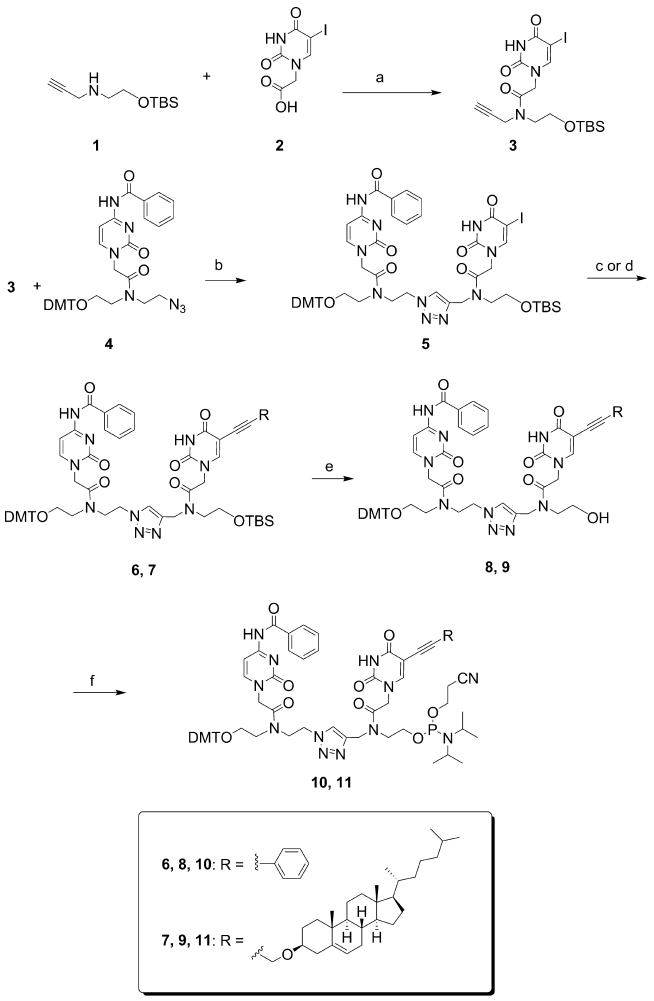


Figure 1. Structural difference of the different modifications used within siRNAs in this study. The $\mathbf{U_tU}$, $\mathbf{C_tU}$, $\mathbf{C_tU^{Ph}}$ and $\mathbf{C_tU^{Ch}}$ modifications correspond to the different triazole-backbone modifications. The **Ch** modification corresponds to the commercially available triethylene glycol cholesterol modification which was purchased as a derivative covalently bound to a controlled-pore glass solid support to conjugate to the 3′-end of the sense strands of siRNAs **aL14** and **aB6**.

To synthesize our siRNAs, our initial efforts were focused on preparing the triazole-based monomer bearing the hydrophobic moieties. This was first initiated via amide-bond coupling of a *tert*-butyldimethylsilyl (TBS)-protected alkyne amine (**1**) to a 5-iodo-uracil-1-yl acetic acid (**2**) with the use of the reagent, *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) to afford the desired compound **3**.

The 4,4′-dimethoxytrityl (DMT)-protected N^4-benzoylcytosinyl-azido compound **4** was prepared from our recently developed protocol.[10] Copper catalyzed azide−alkyne 1,4-cycloaddition (click reaction) of the azide **4** with the alkyne **3** afforded the cycloadduct **5** in good yield.[23,24] The iodinated compound **5** is a substrate for the Sonogashira reaction with a terminal alkyne in the presence of Cu(I) and Pd.[25,26] Two separate reactions involving phenylacetylene and an alkyne-modified cholesterol[27] were completed in excellent yields for compounds **6** (89% yield) and **7** (94% yield). Deprotection of the TBS group of compounds **6** and **7** with tetrabutylammonium fluoride (TBAF) afforded the alcohols **8** and **9**. Phosphitylation of **8** and **9** using 2-cyanoethyl diisopropylchlorophosphoramidite in the presence of diisopropylethylamine (DIPEA) and 4-dimethylaminopyridine (DMAP) afforded the desired phosphoramidites **10** and **11** in good yield. Thus, in relatively few steps, we were able to design two phosphoramidites bearing the desired triazole-backbone unit that contain a hydrophobic moiety from the uracil pyrimidine heterocycle. These unique phosphoramidites were used to site-specifically incorporate modified dinucleotides with a non-phosphate backbone at various positions within the sense strand of different siRNAs that target both the *firefly* luciferase gene and the endogenous gene target *bcl-2*.

Scheme 1. Synthesis of Phosphoramidites[a]



[a]Conditions: (a) 2 equiv of EDC·HCl, DMF, r.t., 61%; (b) 0.5 equiv of $CuSO_4$, 1.6 equiv of sodium ascorbate, THF/H_2O, r.t., 70%; (c) 0.1 equiv of $Pd(PPh_3)_4$, 0.2 equiv of CuI, 2.0 equiv of triethylamine (TEA), 5 equiv of phenylacetylene, 0.13 M DMF, r.t., **6** (89%); (d) 0.1 equiv of $Pd(PPh_3)_4$, 0.2 equiv of CuI, 2.0 equiv of TEA, 5 equiv of alkyne-modified cholesterol, 0.13 M DMF, r.t., **7** (94%); (e) 1.6 equiv of TBAF, THF, r.t., **8** (63%) or **9** (48%); (f) 3 equiv of 2-cyanoethyl diisopropylphosphoramidochloridite, 5.6 equiv of DIPEA, 0.5 equiv of DMAP, dichloromethane (DCM), r.t., **10** (74%) or 11 (64%).

We synthesized a variety of siRNAs that are highlighted in Table 1 through the use of DMT-phosphoramidite chemistry.[28] Figure 1 illustrates the structural differences between the different modifications used within siRNAs in this study.

With the siRNAs that target the luciferase gene, we first examined the silencing potential of our siRNAs in HeLa cells that target a plasmid encoding the *firefly* target gene. HeLa cells were transfected using lipofectamine with two plasmids (the

dx.doi.org/10.1021/ml500260j | ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

Table 1. Sequences of Anti-luciferase (aL) and Anti-bcl-2 (aB) siRNAs[a]

RNA	siRNA duplex
aLwt	5'- CUUACGCUGAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL1	5'- C_tUUACGCUGAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL2	5'- C_tU^{Ph}UACGCUGAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL3	5'- CUUACGC_tUGAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL4	5'- CUUACGC_tU^{Ph}GAGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL5	5'- CUUACGCUCUGUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL6	5'- CUUACGCUC_tU^{Ph}GUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL7	5'- CUUACGCUC_tU^{Ch}GUACUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL8	5'- CUUACGCUGAGUAC_tUUCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL9	5'- CUUACGCUGAGUAC_tU^{Ph}UCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL10	5'- CUUACGCUGAGUAC_tU^{Ch}UCGAtt -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL11	5'- CUUACGCUGAGUACUUCGAU_tU -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL12	5'- CUUACGCUGAGUACUUCGAC_tU^{Ph} -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL13	5'- CUUACGCUGAGUACUUCGAC_tU^{Ch} -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aL14	5'- CUUACGCUGAGUACUUCGAtt**Ch** -3' 3'- ttGAAUGCGACUCAUGAAGCU -5'
aBwt	5'- GUGAAGUCAACAUGCCUGCtt -3' 3'- ttCACUUCAGUUGUACGGACG -5'
aB1	5'- GUGAAGUCAACAUGCC_tU^{Ph}GCtt -3' 3'- ttCACUUCAGUUGUACGGACG -5'
aB2	5'- GUGAAGUCAACAUGCC_tU^{Ch}GCtt -3' 3'- ttCACUUCAGUUGUACGGACG -5'
aB3	5'-GCCUUCUUUGAGUUCGGUGU_tU-3' 3'-ttCGGAAGAAACUCAAGCCAC-5'
aB4	5'- GUGAAGUCAACAUGCCUGCC_tU^{Ph} -3' 3'- ttCACUUCAGUUGUACGGACG -5'
aB5	5'- GUGAAGUCAACAUGCCUGCC_tU^{Ch} -3' 3'- ttCACUUCAGUUGUACGGACG -5'
aB6	5'-GUGAAGUCAACAUGCCUGCtt**Ch** -3' 3'- ttCACUUCAGUUGUACGGACG -5'

[a] U_tU corresponds to the uracil-triazole-uracil modification; C_tU corresponds to the cytosine-triazole-uracil modification; C_tU^{Ph} corresponds to the cytosine-triazole-uracil-phenyl modification; C_tU^{Ch} corresponds to the cytosine-triazole-uracil-cholesterol modification; **Ch** corresponds to the commercial polyethylene-cholesterol modification. 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.

firefly target gene and Renilla, which served as an internal control) and the desired siRNA. Cells were lysed 24 h post-transfection, and the efficacy of the siRNAs was monitored. Figure 2 illustrates the gene-silencing data.

siRNAs **aL1**−**aL4** contained different modifications at varying positions near the 5′-end of the sense strand. In general, these siRNAs exhibited effective dose-dependent downregulation. siRNAs **aL5**−**aL7** contained a variety of substituents at the central region of the duplex. Argonaute 2 is known to cleave the phosphodiester bond of the target mRNA complementary to positions 10 and 11 of the antisense strand.[3,29−31] In the wild-type sequence, the purine motif "GA" occurs at positions 9 and 10. Since our triazole-backbone modification in this study is based on a pyrimidine motif, CU, we examined the effect of replacing the dinucleotide GA at the Argonaute 2 cleavage site of the sense strand with a CU double-mismatch at this position (siRNA **aL5**), and then we asked how both our siRNAs bearing the aforementioned modifications (**aL6 and aL7**) functioned compared to the mismatch. For the double-mismatch siRNA **aL5**, dose-dependent gene-silencing was improved compared to wild-type **aLwt**. This result is consistent with other studies that have introduced mismatches at the central region of siRNAs due to thermal destabilization at the central region.[32] With both siRNAs **aL6** and **aL7**, dose-dependent gene-silencing was observed at levels comparable to wild-type. What is intriguing is that despite the presence of a triazole backbone and the hydrophobic substituents between positions 9 and 10, RNAi activity is well retained. This is encouraging because this result opens the possibility of modifying the central region with a wide variety of functional groups to further fine-tune siRNA potency.

In the next set of experiments, we examined the effect of the modification near the 3′-end of the sense strand. siRNAs **aL8**−**10** were highly effective at gene-silencing, with activity comparable to wild-type **aLwt**. In general, thermal destabilization of the 3′-area of the sense strand of an siRNA duplex often exhibits favorable gene-silencing. siRNAs **aL8**−**10** exhibited a decrease in melting temperature of approximately 14 degrees relative to unmodified wild-type (**aLwt**) siRNA (see Table S2 in Supporting Information). Thermal destabilization of this area of the duplex may offer favorable uptake of the antisense strand to generate an active RISC assembly. In fact, the IC_{50}'s for **aL9** and **aL10** were 4.53 and 6.52 pM, respectively, indicative of effective gene-silencing. Finally, we examined the effect of triazole-modified derivatives at the 3′-end of the sense strand. siRNAs **aL11**, **aL12**, and **aL13** were highly potent, and each had an IC_{50} of 1.43 pM, 1.26 pM, and 1.28 pM, respectively. In comparison, the siRNA that had the commercially available triethylene glycol cholesterol at the 3′-end (**aL14**) had an IC_{50} comparable to wild-type siRNA (3.23 pM) (see Figure S3 in Supporting Information for IC_{50}'s for siRNAs **aLwt**, **aL4**, and **aL9**−**14**). The enhanced potency of our siRNAs **aL12** and **aL13** suggests that the triazole-backbone core with the hydrophobic modifications at the 3′-end enhances the activity of the siRNA compared to wild-type **aLwt**. Previous work from our laboratory has shown that the triazole-backbone unit at this position offers enhanced stability to serum nucleases, which is likely a factor in the observed increase in potency for these siRNAs, **aL12** and **aL13**.[10]

One of the current limitations of siRNAs includes its poor ability to permeate across cellular membranes. Many efforts have been placed forward to modify siRNAs with hydrophobic moieties to mitigate some of the challenges in delivery. We examined the effect of our siRNA in the absence of the carrier typically used in *in vitro* cell-based studies, lipofectamine, on

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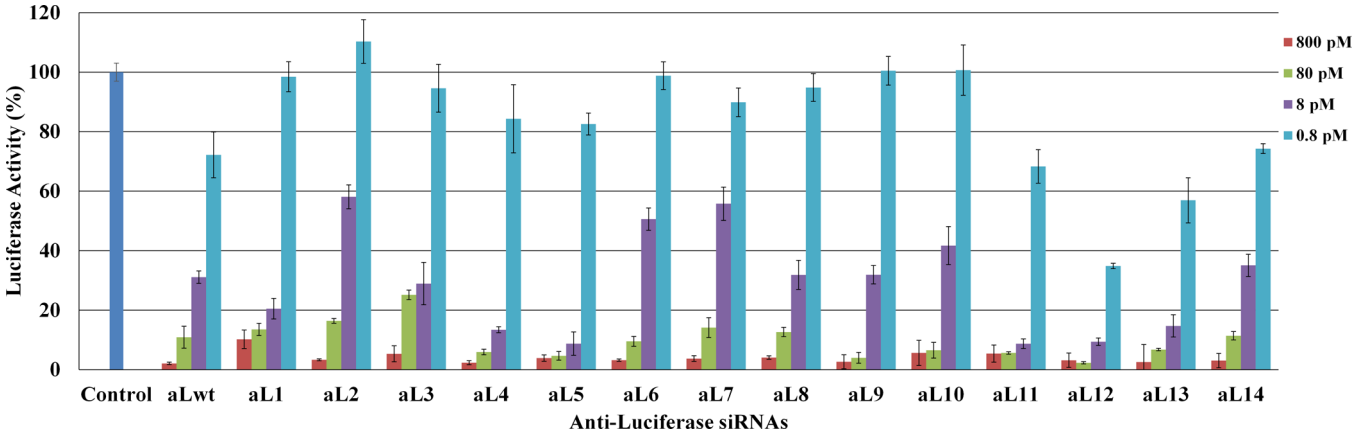

Figure 2. Reduction in *firefly* luciferase expression related to the potency of chemically modified siRNAs using the Dual-luciferase reporter assay. The siRNAs were tested at 0.8, 8, 80, and 800 pM, with *firefly* luciferase expression normalized to *Renilla* luciferase.

exerting a gene-silencing effect at 50, 200, and 3000 nM. The siRNAs tested (**aLwt** and **aL12**−**aL14**) exhibit dose-dependent gene-silencing against the *firefly* luciferase target. One of the most exciting effects as illustrated in Figure 3 is that the siRNA

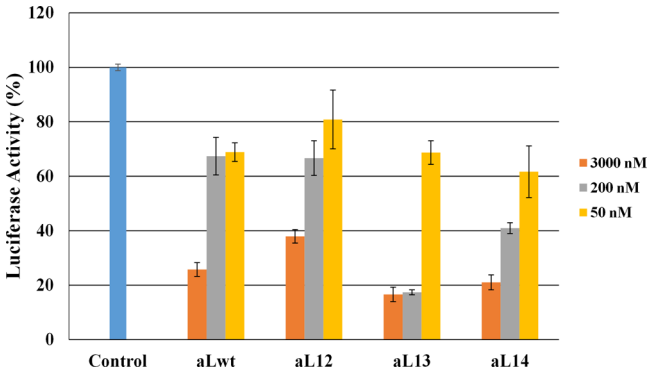

Figure 3. Reduction in *firefly* luciferase expression in the absence of the carrier lipofectamine. Chemically modified siRNAs were tested at 50, 200, and 3000 nM with *firefly* luciferase expression normalized to *Renilla* luciferase.

with the C_tU^{Ch} modification at the 3′-end (**aL13**) exhibited the best gene-silencing (ca. 80% reduction in luciferase activity) among those tested at 200 nM. When compared to wild-type siRNA (**aLwt**) and **aL12**, only approximately 35% down-regulation was observed at this concentration. The siRNA bearing the commercially available 3′-cholesterol modification (**aL14**) exhibited improved activity compared to wild-type siRNA **aLwt** (ca. 60% knockdown), at 200 nM, perhaps due to enhanced cell-membrane permeability. Our cholesterol-modified siRNA **aL13** showed the greatest promise in its ability to efficiently knockdown gene expression in a carrier-free assay. In addition, cell proliferation assay experiments of our siRNAs **aL12**−**aL14** exhibit very little cytotoxicity between 50 and 200 nM; however, the wild-type **aLwt** exhibits some degree of toxicity. This suggests that our chemically modified siRNAs are downregulating gene expression in the absence of a carrier complex. In addition, it appears that the siRNAs used (**aL12**−**aL14**) are not as cytotoxic compared to wild-type **aLwt** (even at high concentration of 3000 nM), thus suggesting that the modified siRNAs are providing a means to reduce overall cytotoxicity (see Figure S1 in Supporting Information).

We wanted to explore whether our siRNAs would hold promise against the endogeneous chromosomal target bcl-2. Bcl-2 has shown promise as an anticancer target, as it is a key regulatory protein involved in cellular apoptosis.[33,34] We synthesized siRNAs bearing the C_tU^{Ph} and C_tU^{Ch} modification at position 16−17 of the sense strand (**aB1** and **aB2**). In addition, we synthesized siRNAs containing the U_tU, C_tU^{Ph}, C_tU^{Ch}, and **Ch** modification at the 3′-position (**aB3**−**aB6**) of the sense strand. HeLa cells were transfected using lipofectamine with the desired siRNA. The ability of these siRNAs to downregulate bcl-2 mRNA levels was assessed 24 h post-transfection in cells by measuring normalized expression levels using real-time polymerase chain reaction (RT-qPCR) (see Supporting Information for conditions). The data from Figure 4 illustrates that our chemically modified siRNAs show effective

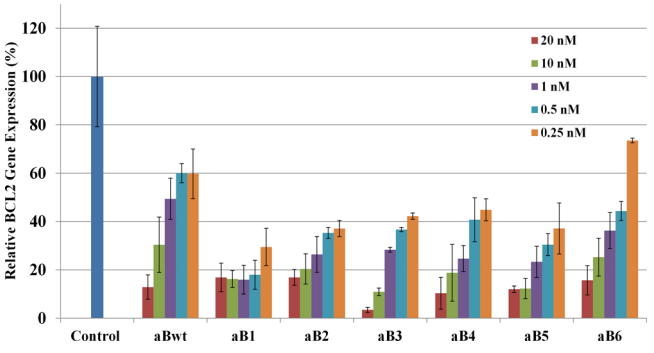

Figure 4. Reduction in *bcl-2* expression in the presence of chemically modified siRNAs. The siRNAs were tested at 0.25, 0.5, 1, 10, and 20 nM with *bcl-2* expression normalized to 18s RNA.

dose-dependent downregulation of *bcl-2* at 0.25, 0.5, 1, 10, and 20 nM. In fact, siRNAs **aB1** and **aB2** exhibit activity that is enhanced when compared to unmodified wild-type siRNA, **aBwt**, at concentrations at 10 nM and below. Comparing the 3′-modifications, both **aB4** and **aB5** exhibit outstanding gene-silencing of *bcl-2*, which appears to be more effective than **aBwt**. In addition, **aB3** contains a U_tU modification at the 3′-end, and this siRNA also exhibits potent gene-silencing. Finally, the siRNA bearing the commercially available cholesteryl modification, **aB6**, was not as effective as the modifications used in this study, thus highlighting the broad utility and greater

dx.doi.org/10.1021/ml500260j | *ACS Med. Chem. Lett.* XXXX, XXX, XXX−XXX

efficiency of our 3′-modified siRNA bearing the $\mathbf{C_tU^{Ch}}$ modification.

Our siRNAs used in this study exhibit excellent gene-silencing characteristics in both exogeneous and endogeneous gene systems. The modifications used in this study that are located at the 3′-end of the sense strand exhibit superior gene-silencing compared to wild-type siRNAs. Furthermore, studies from our laboratory involving triazole-linked siRNAs have shown that these modifications are thermally destabilizing when located internally.[10] In fact, in this study both the phenyl and the cholesterol group do not appear to impact the melting temperature of the duplexes when compared to similar chemically modified siRNAs bearing a $\mathbf{C_tU}$ or a $\mathbf{U_tU}$ modification.[10] Our studies with several of our modified strands indicate that these modifications are also destabilizing when compared to wild-type (see Table S2 in the Supporting Information). In addition, these siRNAs exhibit a classical A-type conformation, as illustrated by negative and positive circular dichroism bands at approximately 210 and 265 nm, respectively (see Figure S2 in the Supporting Information). This highlights that its biophysical properties are amenable for substrate recognition by RISC.

In conclusion, we are reporting the effective gene-silencing properties of triazole-backbone modified siRNAs that contain hydrophobic groups appended on the 5 position of the uracil pyrimidinyl group. We utilized both an endogeneous target and an exogenous target. Both systems indicate that our substrate is an effective substrate for the RNAi pathway. Enhancement in activity is observed with an siRNA that contains a $\mathbf{C_tU^{Ch}}$ modification in the absence of the carrier complex, lipofectamine, thus highlighting the potential impact of the development of carrier-free delivery of siRNAs through this conjugation. Future work includes determining the mechanism and mode of action of our siRNAs in carrier-free environments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, along with copies of ^{1}H and ^{13}C NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

Funding

We acknowledge the National Sciences and Engineering Research Council and the Canada Foundation for Innovation for funding.

Notes

The authors declare no competing financial interest.

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