

Targeted delivery and enhanced gene-silencing activity of centrally modified folic acid–siRNA conjugates

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ABSTRACT

One of the major hurdles in RNAi research has been the development of safe and effective delivery systems for siRNAs. Although various chemical modifications have been proposed to improve their pharmacokinetic behaviour, their delivery to target cells and tissues presents many challenges. In this work, we implemented a receptor-targeting strategy to selectively deliver siRNAs to cancer cells using folic acid as a ligand. Folic acid is capable of binding to cell-surface folate receptors with high affinity. These receptors have become important molecular targets for cancer research as they are overexpressed in numerous cancers despite being expressed at low levels in normal tissues. Employing a post-column copper-catalyzed alkyne–azide cycloaddition (CuAAC), we report the synthesis of siRNAs bearing folic acid modifications at different positions within the sense strand. In the absence of a transfection carrier, these siRNAs were selectively taken up by cancer cells expressing folate receptors. We show that centrally modified folic acid–siRNAs display enhanced gene-silencing activity against an exogenous gene target (~80% knockdown after 0.75 μ M treatment) and low cytotoxicity. In addition, these siRNAs achieved potent dose-dependent knockdown of endogenous Bcl-2, an important anti-apoptotic gene.

INTRODUCTION

RNA interference (RNAi) is an endogenous pathway that utilizes double-stranded RNA to suppress the expression of a target mRNA, resulting in sequence-specific gene silencing (1,2). In the effector step of RNAi, short interfering RNAs (siRNAs) of 21–23 nucleotides are incorporated into a protein complex, the RNA-induced silencing complex (RISC) (3). This is followed by a duplex dissociation step, promoted by the catalytic activity of the endonuclease

Argonaute2 (Ago2) which cleaves between base pairs 9 and 10 from the sense strand 5' end (4,5). RISC retains the anti-sense strand which is used as a guide sequence to locate and degrade the target mRNA (6,7). Synthetic siRNAs are able to induce gene silencing through the RNAi pathway (8), becoming powerful tools to study gene function (9,10). RNAi-based therapies also hold great promise as siRNAs can be used to down-regulate the expression of deleterious proteins involved in disease onset and progression (11–13). However, this system comes with several limitations given by the inherent nature of siRNAs such as low stability, poor cellular uptake, potential for immune activation and off-target effects (14–16). Chemical modifications are able to mitigate some of these challenges and improve the pharmacokinetic properties of siRNAs (17,18) but despite advancements in the field (19), there is still no universal modification able to address all of the challenges associated with siRNAs.

The delivery of siRNAs to target cells or tissues has been one of the major challenges in RNAi research. Naked siRNAs are unable to diffuse across cellular membranes due to their large size and polyanionic backbone (20). Current delivery strategies include the encapsulation of siRNAs within nanoparticles or liposomes and the conjugation of siRNAs to hydrophobic molecules (21). Because siRNAs lack selectivity for specific cell types, receptor-targeting ligands can be used to deliver siRNAs to target cells and tissues (22). One example is the vitamin folic acid, which has been extensively used as a drug delivery system to target folate receptors (FRs) in tumour cells (23,24). FRs are cell-surface glycoproteins able to bind folic acid with high affinity. These receptors are expressed at low levels in most tissues, as their expression is limited to cells important for folate resorption and embryonic development, yet they are highly overexpressed on the surface of numerous cancers (25). This includes ~90% of ovarian carcinomas as well as breast, endometrial, brain and kidney cancers (26,27). Once bound to the FR, folic acid enters the cell through receptor-mediated endocytosis. Notably, folic acid conjugates retain the ability to bind to and be internalized by this receptor, making the FR an attractive molecular target for cancer research (28). This receptor-targeting strategy has been used to de-

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liver siRNAs by functionalizing liposomes and nanoparticles with folic acid (29–33) although selective delivery can also be achieved by direct conjugation of folic acid to siRNAs.

Previous studies have successfully incorporated folic acid modifications at either the 3' or 5' end of siRNA and achieved selective, carrier-free delivery to target cells (34,35). In these studies, moderate gene-silencing activity against exogenous gene targets (40–60% knockdown after 1 μ M treatment) was reported. These results show promise in the use of folic acid as a delivery system for siRNAs. However, there is a need to improve the gene-silencing potency of folic acid–siRNA constructs. Recently, our lab group reported a method to destabilize the central region of siRNAs, which spans the Ago2 cleavage site. We showed that chemical modifications within this region can lead to potent gene-silencing (36,37). To the best of our knowledge, folic acid has not been incorporated into the central region of siRNAs. Based on this, we report the copper-catalyzed azide-alkyne cycloaddition (CuAAC) synthesis of siRNAs bearing folic acid modifications at different positions within the sense strand, with a particular emphasis on the central region. In the absence of a transfection carrier, these siRNAs were selectively taken up by FR-expressing cell lines. We show that internal modified folic acid–siRNAs display enhanced gene-silencing activity, with minimal toxicity, against exogenous firefly luciferase mRNA (~80% knockdown after 0.75 μ M treatment). In addition, these siRNAs achieved potent dose-dependent knockdown of the oncogene Bcl-2 (~72% knockdown after 1 μ M treatment).

MATERIALS AND METHODS

General methodology and materials as well as NMR spectra are provided in the Supplementary Data.

Synthesis of propargyl phosphoramidite

Compound 1. To a solution of diethanolamine (10 g, 95.1 mmol) in 150 ml of anhydrous CH_2Cl_2 , cooled in an ice bath, was added anhydrous potassium carbonate (65.7 g, 0.476 mol) under an argon atmosphere. Propargyl bromide (80 wt% in toluene, 8.5 ml, 95.1 mmol) was added drop-wise over a 5-minute period and the solution was left to stir vigorously for 60 h at room temperature. The crude product was filtered to remove the potassium carbonate and the collected filtrate was concentrated *in vacuo* to produce a dark amber oil, which was purified by flash chromatography (elution with 2 to 10% MeOH/ CH_2Cl_2). The final product was isolated as a clear amber oil (9.53 g, 70%). ^1H NMR (400 MHz, CDCl_3) δ 2.19 (t, 1H), 2.68 (t, 4H), 3.40 (s, 2H), 3.60 (t, 4H). ^{13}C NMR (101 MHz, CDCl_3) δ 42.05, 55.21, 59.05, 73.15, 78.31. (Scheme 1)

Compound 2. To a solution of **1** (2 g, 14 mmol) in 25 ml anhydrous CH_2Cl_2 was added freshly-distilled triethylamine (1.7 ml, 12.6 mmol) under an argon atmosphere. This was followed by the drop-wise addition of 4,4'-dimethoxytriphenylmethyl chloride (3.79 g, 11.2 mmol) in 5 ml anhydrous CH_2Cl_2 . The reaction mixture was stirred overnight at room temperature after which the crude product was extracted three times with a saturated NaHCO_3

solution. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to produce a cloudy yellow oil which was purified by flash chromatography (elution with 2 to 10% MeOH/ CH_2Cl_2). The final product was isolated as a clear yellow oil (2.8 g, 45%). ^1H NMR (400 MHz, CDCl_3) δ 2.23 (t, 1H), 2.75 (t, 2H), 2.83 (t, 2H), 3.23 (t, 2H), 3.44(d, 2H), 3.60 (t, 2H), 3.80 (s, 6H), 6.87 (dt, 4H), 7.29 (m, 1H), 7.31 (td, 2H), 7.38 (dt, 4H), 7.47 (d, 2H); ^{13}C NMR (101 MHz, CDCl_3) δ 42.76, 52.58, 55.19, 55.65, 58.57, 61.94, 72.83, 78.82, 86.21, 113.07, 126.71, 127.77, 128.13, 129.96, 136.28, 144.95, 158.41.

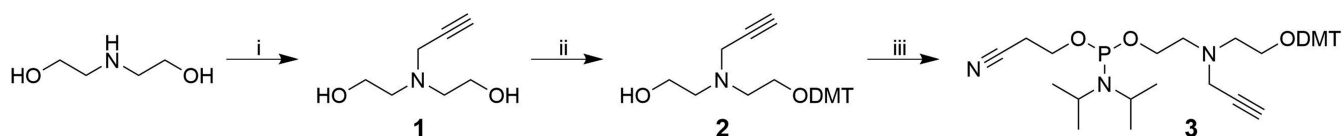
Compound 3. To a flame-dried round-bottomed flask containing a solution of **2** (180 mg, 0.404 mmol) in 5 ml anhydrous CH_2Cl_2 was added freshly-distilled triethylamine (0.28 ml, 2.02 mmol) under an argon atmosphere. This was followed by the dropwise addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.27 ml, 1.21 mmol). The reaction mixture was stirred for 3 h and then concentrated *in vacuo* to produce a cloudy oil which was purified by flash chromatography (elution 20–60% EtOAc/hexanes, maintaining 5% triethylamine). The product was isolated as a clear oil (0.22 g, 84%). ^1H NMR (400 MHz, CDCl_3) δ 1.17 (dd, 12H), 2.19 (t, 1H), 2.55 (m, 2H), 2.82 (dt, 4H), 3.15 (t, 2H), 3.45 (d, 2H), 3.57 (m, 2H), 3.66 (m, 2H), 3.76 (m, 2H), 3.78 (s, 6H), 6.83 (dt, 4H), 7.20 (tt, 1H), 7.27 (t, 2H), 7.32 (dt, 4H), 7.44 (d, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 20.33, 21.02, 24.63, 42.98, 43.10, 46.30, 54.20, 55.15, 58.41, 62.03, 62.65, 72.79, 79.27, 86.05, 113.04, 117.71, 126.58, 127.75, 128.18, 130.02, 136.46, 145.22, 158.36. ^{31}P (162 MHz, CDCl_3) δ 147.28.

Oligonucleotide synthesis, deprotection and purification

Wild-type and propargyl oligonucleotides were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer using a 1.0 μ M dT controlled-pore glass (CPG) support and a 1.0 μ M cycle with a 999-second coupling time. Immediately prior to synthesis, phosphoramidites were resuspended in anhydrous acetonitrile to a final concentration of 0.1 M. Oligonucleotide sense strands were chemically phosphorylated at the 5' end using 2-[2-(4,4-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite. Cleavage of oligonucleotides from the solid support was achieved by flushing the CPG columns with 1 ml EMAM solution (1:1 methylamine 33 wt% in ethanol and methylamine 40% wt. in H_2O) for 1 h at room temperature, followed by overnight incubation in EMAM to deprotect the bases. Oligonucleotides were concentrated in a miVac Quattro concentrator and desilylated in DMSO (100 μ l) and 3HF-Et₃N (125 μ l) for 3 h at 65°C. Crude oligonucleotides were precipitated in ethanol and desalted using Millipore Amicon Ultra 3000 MW cellulose centrifugal filters. Strands were purified using reverse-phase HPLC eluting from 5% to 95% ACN in 0.1 M TEAA buffer (pH 7.0).

Synthesis and purification of folic acid–conjugated siRNAs

Compound 4. Folic acid (0.5 g, 1.13 mmol) was dissolved in anhydrous DMSO (30 ml) under an argon atmosphere. *N*-Hydroxysuccinimide (0.26 g, 2.27 mmol) and



Scheme 1. Reagents and conditions: (i) propargyl bromide, K_2CO_3 , CH_2Cl_2 , 60 h, 69%; (ii) DMTCl, Et_3N , CH_2Cl_2 , rt, overnight, 45%; (iii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, Et_3N , CH_2Cl_2 , rt, 3 h, 84%.

N,N'-dicyclohexylcarbodiimide (0.26 g, 1.25 mmol) were simultaneously added and the reaction mixture was left to stir overnight in the dark. The dicyclohexylurea by-product was removed by filtration and the filtrate was collected in a round-bottomed flask to which a solution 2-azidoethanamine (0.12 g, 1.37 mmol) in 10 ml anhydrous DMSO was added. The reaction mixture was left to stir in the dark for an additional 24 h. After removing most of the DMSO *in vacuo*, the crude product was precipitated in cold diethyl ether and the collected yellow crystals were washed with THF and CH_2Cl_2 . The product was further purified by automated flash chromatography, eluting with a slow gradient (0–100%) of solution A (2:1:1 $NH_4OH/MeOH/ACN$) to solution B (ACN). The product was isolated as bright yellow crystals (0.48 g, 84%). 1H NMR (400 MHz, $DMSO-d_6$) δ 1.84–2.00 (m, 2H), 2.05–2.20 (m, 2H), 3.19 (t, 2H), 3.32 (t, 2H), 4.30 (m, 1H), 4.45 (d, 2H), 6.61 (d, 2H), 6.90 (m, 1H), 7.66 (d, 2H), 8.04 (m, 1H), 8.59 (s, 1H), 11.86 (br, 1H). ESI-HRMS (ES+) *m/z* calculated for $C_{21}H_{23}N_{11}O_5$: 510.1956, found 510.1953 $[M+H]^+$ (Scheme 2).

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) procedure. To a solution of propargyl-modified RNA (100 μM , 5 μl) in $DMSO/H_2O/t-BuOH$ (1:2:1) was added 4 (2.5 mM, 5 μl) under an argon atmosphere. This was followed by the addition of a pre-chelated mixture of $CuSO_4$ (2.5 mM) and tris(benzyltriazolylmethyl)amine (TBTA) (12.5 mM, 5 μl). A fresh solution of sodium ascorbate (2.5 mM, 10 μl) was added, and the reaction mixture was stirred at 40°C in the dark for 3.5 h. At this point, sodium acetate (0.3 M solution in H_2O , 50 μl) was added and the mixture was stirred for an additional 20 min at room temperature. Crude RNA was precipitated in cold EtOH, centrifuged at 13 400 rpm for 15 min and washed twice with cold EtOH. Strands were purified using reverse-phase HPLC eluting from 5% to 95% ACN in 0.1 M TEAA buffer (pH 7.0). (Scheme 3)

Thermal denaturation and CD studies

Thermal denaturation and CD studies were performed using a Jasco J-815 Circular Dichroism (CD) Spectropolarimeter equipped with a temperature controller. For duplex formation, equimolar amounts of complementary sequences were combined, dried down and resuspended in 300 μl pH 7 sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na_2HPO_4 , 1.00 mM EDTA). Samples were heated at 90°C for 2 min and then allowed to slowly cool to room temperature. To determine melting temperature (T_m), UV absorbance was measured at 260 nm and temperature was increased from 10 to 95°C at a rate of 0.5°C per minute. T_m data was analysed using Meltwin v3.5 software and represents the average of three independent runs. Circular dichro-

ism spectra were recorded at 25°C, scanning from 200 to 350 nm with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. All scans were performed in triplicate and averaged using Jasco's Spectra Manager v2 software.

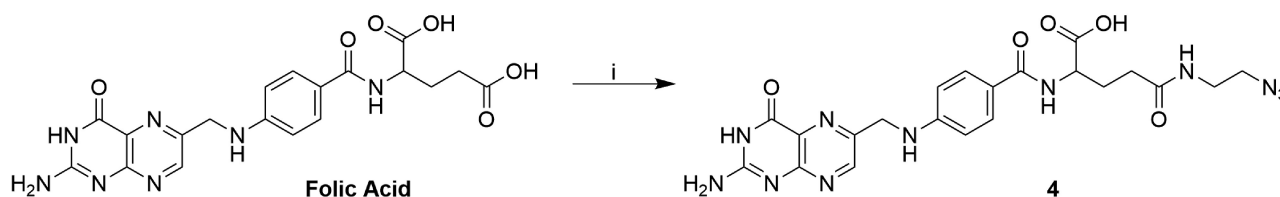
Cell culture

HeLa and HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 Medium respectively, both supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and passaged at 80% confluency.

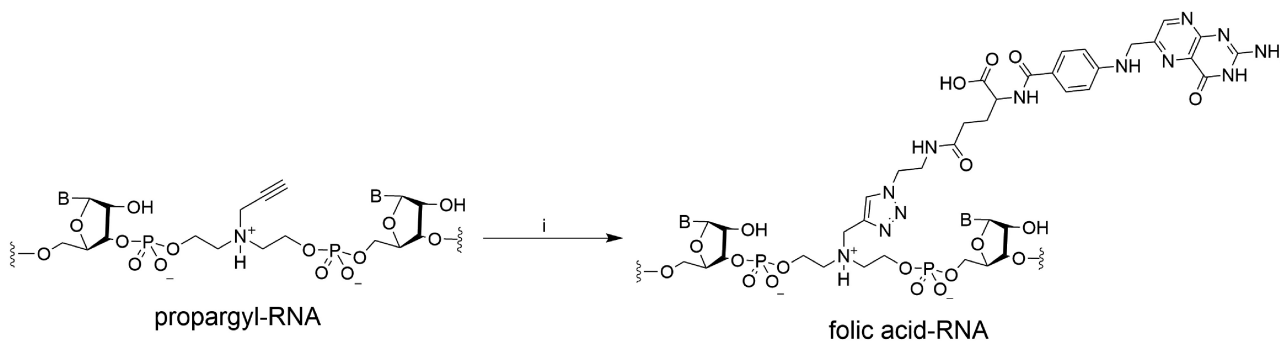
Carrier-free transfections for luciferase assay

Transfection in HeLa. The day before transfection, HeLa cells were seeded into 96-well plates, containing 50 μl folate-free RPMI 1640, at a density of 1.0×10^4 cells per well and incubated for 24 h. Two plasmids, pGL3 (firefly luciferase, 200 ng) and pRLSV40 (*Renilla* luciferase, 50 ng), were co-transfected using 1 μl Lipofectamine 2000™ (Invitrogen) following the manufacturer's protocol. Plates were incubated for 4 hours at 37°C in a humidified atmosphere with 5% CO_2 after which the medium was removed from each well. Cells were washed twice with 1 \times phosphate-buffered saline (PBS) after which 50 μl folate-free RPMI 1640 medium (without antibiotics) was added to each well. Each siRNA was diluted in 50 μl 1 \times Gibco's Opti-MEM Reduced Serum Medium (Invitrogen) on ice and the diluted samples were immediately transferred to the respective wells of the 96-well plate. Plates were gently rocked back and forth for a few minutes and then incubated for an additional 16 h prior to cell lysis.

Transfection in HT-29. The day before transfection, HT-29 cells were seeded into 96-well plates, containing 50 μl folate-free RPMI 1640, at a density of 5.0×10^4 cells per well and incubated for 24 h. For plasmid transfection, pGL3 (firefly luciferase, 600 ng) and pRLSV40 (*Renilla* luciferase, 150 ng) were combined and diluted in 1 \times Gibco's Opti-MEM Reduced Serum Medium (Invitrogen) to a final volume of 50 μl . This was followed by the addition of 4 μl Lipofectamine® LTX (Thermo Fisher). After a 30-minute incubation period at room temperature, complexes were transferred to each well and plates were incubated for 6 h at 37°C in a humidified atmosphere with 5% CO_2 after which the medium was removed from each well. Cells were washed twice with 1 \times phosphate-buffered saline (PBS) after which 50 μl folate-free RPMI 1640 medium (without antibiotics) was added to each well. Each siRNA was diluted in 50 μl



Scheme 2. Reagents and conditions: (i) 3, *N*-Hydroxysuccinimide, *N,N'*-Dicyclohexylcarbodiimide, DMSO, rt, overnight, 2-azidoethanamine, 24 h., 83%.



Scheme 3. Reagents and conditions: 4, CuSO₄, TBTA, sodium ascorbate, DMSO/H₂O/*t*-BuOH (1:2:1), 40°C, 4 h, 69–80%

1× Gibco's Opti-MEM Reduced Serum Medium (Invitrogen) on ice and the diluted samples were immediately transferred to the respective wells of the 96-well plate. Plates were gently rocked back and forth for a few minutes and then incubated for an additional 20 h prior to cell lysis.

Dual-luciferase[®] reporter assay

Cells were lysed with 1× passive lysis buffer for 20 min at room temperature. Cell lysates were transferred to microcentrifuge tubes and were immediately used to assess the gene-silencing activity of siRNAs using a Dual-Luciferase[®] Reporter Assay (Promega). Luciferase Assay Reagent II (LAR II) and Stop & Glo[®] Reagent were prepared following the manufacturer's protocol. Cell lysates (10 μl) were transferred to Costar 96-well plates in triplicate. LAR II reagent (50 μl) was added to each well and the first luminescence measurement was taken on a Synergy HT (Bio-Tek) plate luminometer. Stop & Glo[®] Reagent (50 μl) was then added to each well and the second luminescence measurement was taken. Results are expressed as the ratio of firefly/*Renilla* luminescence taken as a percentage of an untreated control. Each value is the average of at least three biological replicates and error bars indicate standard deviation.

Statistical analysis

Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used to generate dose-response curves after carrier-free siRNA transfection in HeLa cells. Dose-response curves for folic acid-conjugated anti-luciferase siRNAs can be found in the Supplement (Supplementary Figure S2). The half-maximal inhibitory concentration (IC₅₀) of each siRNA was determined using Prism's variable slope (four-parameter) model. Anti-luciferase siRNAs were tested at

seven concentrations in carrier-free conditions. Anti-Bcl-2 siRNAs were tested at three concentrations in carrier-free conditions. Standard errors (S.E.) were determined for a minimum of two biological replicates.

Cell viability assay

HeLa and HT-29 cells were seeded into 96-well plates at a density 5.0 × 10³ cells per well and incubated for 24 h. Cells were transfected following the described carrier-free protocol and were incubated for an additional 24 h. Cell viability was assessed using the XTT Cell Proliferation Assay Kit (ATCC[™]) following the manufacturer's protocol. Absorbance readings were taken using a Synergy HT (Bio-Tek) plate luminometer.

Flow cytometry

PE anti-FOLR1 (Folate Binding Protein) Antibody and PE Mouse IgG2a, κ Isotype Control (FC) Antibody were purchased from Biolegend. Cells were dislodged from the culture flask using trypsin and transferred into tubes. Cells were then centrifuged at 300 g for 10 min (4°C). After aspirating the supernatant, cells were resuspended in 150 μl cell staining buffer (2.5 ml FBS, 47.5 ml PBS). Cells were stained with trypan blue and counted using a Haemocytometer. For each study, cells were resuspended in staining buffer to achieve a final concentration of 1 × 10⁶ cells/100 μl. Antibodies were added to each cell suspension and cells were incubated in the dark for 30 min (on ice). Samples were centrifuged at 300 g for 5 min (4°C) after which the supernatant was aspirated and cells were washed with 1 ml staining buffer. The last two steps, centrifugation and washing, were repeated once more. Samples were then centrifuged one last time. After removing the supernatant, cells were resuspended in 500 μl ice-cold

PBS and incubated at room temperature for 5 min. Flow cytometry studies were performed immediately on a BD Accuri C6 Plus flow cytometer following the manufacturer's protocol.

RT-qPCR

Detailed methods for total RNA extraction and cDNA synthesis can be found in the Supplementary Data. Real-time PCR was performed in a total reaction volume of 20 μ l including 10 μ l SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) containing Sso 7-d Fusion Polymerase, 0.5 μ M forward primer and reverse primer and 2 μ l cDNA template. In the final reaction, cDNA was diluted 40 \times to produce the best results. Pre-designed primers BCL-2F 5'-CTG GTG GGA GCT TGC ATC AC-3' and BCL-2R 5'-ACA GCC TGC AGC TTT GTT TC-3' were purchased to target the Bcl-2 gene and yielding a 150-bp amplicon and 18S-F 5'-CGG CTA CCA CAT CCA AGG AAG-3' and 18S-R 5'-CGC TCC CAA GAT CCA ACT ACT-3' (Integrated DNA Technologies Inc, San Diego, California) were used to target the 18s gene in HeLa cells and yielding a 247-bp amplicon. Reactions were incubated in the Bio-Rad CFX 96 Real-Time Detection System using the following cycle conditions: 50°C for 10 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Reaction specificity was assessed by melting curve analysis immediately after the qPCR experiment. The efficiency of each primer set for RT-qPCR was determined to be between 95 and 100% using the standard curve method. NTC controls were performed during standard curve analysis to confirm that amplification of the PCR product was cDNA and not genomic DNA. NTC controls were also performed to ensure that amplification of the PCR product was not a result of primer-dimers. Results were analyzed using the Bio-Rad CFX manager 3.1 software where the Bcl-2 expression data was normalized against 18s gene as the reference and expression profiles were generated using the comparative Delta-CT method of analysis. The final data was represented by averages and standard deviations compiled from two biological replicates for each treatment for which three technical replicates were included for the qPCR experiments.

RESULTS

Preparation of propargyl and folic acid-modified oligonucleotides

Propargyl phosphoramidite **3** was synthesized as described above. Propargyl and wild-type oligonucleotides were synthesized following standard solid-phase phosphoramidite chemistry. Propargyl-modified oligonucleotides were conjugated with azido-folate derivative **4** as described above. Oligonucleotides were purified using reverse-phase HPLC (Supplementary Figure S5) and characterized by mass spectrometry (Supplementary Table S1).

Thermal stability and CD studies

Synthesized sense strands were annealed to their complementary antisense sequences as described above. CD studies were performed to confirm that siRNAs adopted an A-form

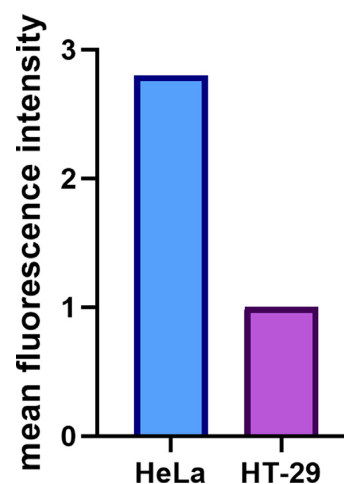


Figure 1. Relative expression of folate receptor α (FR α) levels in HeLa and HT-29 cells determined by flow cytometry.

helical conformation (Supplementary Figure S1). Melting temperatures (T_m) were measured for anti-firefly luciferase and anti-Bcl-2 siRNAs (Table 1). Modifications placed at the 3' end of the sense strand were well-tolerated and did not cause significant destabilizing effects. In these siRNAs, the propargyl and folic acid modifications replaced the 3' dTdT overhang, leading to a 3.5 and 4.7°C decrease in melting temperature for aL-P4 and aL-F4, respectively. This is likely due to the loss of stacking interactions which have been reported with 3' dTdT overhangs (38). We observed a similar destabilizing effect when the modifications were placed at position 5 from the sense strand 3' end ($\Delta T_m = -6.00$ and -5.5°C for propargyl and folic acid-siRNAs, respectively). On the other hand, internally-modified anti-luciferase siRNAs exhibited significant thermal destabilization. The greatest decrease in T_m was observed when the propargyl spacer replaced a single nucleotide at position 9 from the sense strand 5' end (aL-P1, $\Delta T_m = -22.2^\circ\text{C}$). Placing the folic acid modification at this position produced a similar effect (aL-F1, $\Delta T_m = -17.2^\circ\text{C}$). This thermal destabilization is consistent with previous studies examining the effect of central region modifications on siRNA stability (39). Next, we tested whether our propargyl and folic acid modifications would be better accommodated within the helix if they replaced two nucleotides instead of one (positions 9 and 10 from the sense strand 5' end). However, there was no significant increase in melting temperature ($\Delta T_m = -20.5$ and -19.9 for aL-P2 and aL-F2 siRNAs respectively). Similar destabilizing effects were observed with internal modified anti-Bcl-2 siRNAs (aB-P and aB-F) and scramble siRNAs (aL-scr-P, aL-scr-F, aB-scr-P and aB-scr-F).

Relative expression of folate receptor α in HeLa and HT-29 cells

The relative expression of cell-surface folate receptor α (FR α) was assessed in HeLa and HT-29 cells using flow cytometry. The procedure was performed as described above and results are summarized in Figure 1. HeLa cells dis-

Table 1. siRNA sequences, melting temperatures and *IC₅₀ values

siRNA	Duplex	T _m (°C)	ΔT _m (°C)	IC ₅₀ ± S.E. (nM)
aL-wt	5' CUUACGCUGAGUACUUCGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	76.1	–	Inactive
aL-P1	5' CUUACGCUPAGUACUUCGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	53.9	–22.2	Inactive
aL-F1	5' CUUACGCUFAGUACUUCGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	58.9	–17.2	171.0 ± 48.8
aL-P2	5' CUUACGCUPGUACUUCGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	55.6	–20.5	Inactive
aL-F2	5' CUUACGCUFGUACUUCGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	56.2	–19.9	128.95 ± 9.7
aL-P3	5' CUUACGCUGAGUACUPGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	70.1	–6.0	Inactive
aL-F3	5' CUUACGCUGAGUACUFGAtt 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	70.6	–5.5	283.9 ± 62.9
aL-P4	5' CUUACGCUGAGUACUUCGAP 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	72.6	–3.5	Inactive
aL-F4	5' CUUACGCUGAGUACUUCGAF 3' 3' ttGAAUGCGACUCAUGAAGCU 5'	71.4	–4.7	1044 ± 23.0
aL-scr	5' GGUAUCCUCGUGAAUCAUtt 3' 3' ttCCAUAGGGAGCACUUAGUA 5'	74.5	–	Inactive
aL-scr-P	5' GGUAUCCCPGUGAAUCAUtt 3' 3' ttCCAUAGGGAGCACUUAGUA 5'	59.1	–15.4	Inactive
aL-scr-F	5' GGUAUCCCFGUGAAUCAUtt 3' 3' ttCCAUAGGGAGCACUUAGUA 5'	54.6	–19.9	Inactive
aB-wt	5' GCCUUCUUUGAGUUCGGUGtt 3' 3' ttCGGAAGAAACUCAAGCCAC 5'	72.8	–	Inactive
aB-P	5' GCCUUCUUUPAGUUCGGUGtt 3' 3' ttCGGAAGAAACUCAAGCCAC 5'	56.6	–16.2	Inactive
aB-F	5' GCCUUCUUUFAGUUCGGUGtt 3' 3' ttCGGAAGAAACUCAAGCCAC 5'	47.6	–25.2	419.3
aB-scr	5' GGUGUACGUCGUCUGUUCUtt 3' 3' ttCCACAUGCAGCAGACAAGA 5'	73.1	–	Inactive
aB-scr-P	5' GGUGUACGPGUCUGUUCUtt 3' 3' ttCCACAUGCAGCAGACAAGA 5'	67.1	–6.0	Inactive
aB-scr-F	5' GGUGUACGFGUCUGUUCUtt 3' 3' ttCCACAUGCAGCAGACAAGA 5'	55.0	–18.1	Inactive

The top strand corresponds to the sense strand; the bottom strand corresponds to the antisense strand. **P** corresponds to the propargyl spacer. **F** represents the folic acid spacer. The Argonaute2 cleavage site is underlined. **aL** represents anti-luciferase siRNAs. **aB** represents anti-Bcl-2 siRNAs.

*IC₅₀ values were calculated after siRNA transfection in a carrier-free environment. Inhibitory dose-response curves can be found in the Supplementary Data (Supplementary Figure S2).

played a 3-fold increase in FR α expression compared to HT-29 cells.

Carrier-free gene silencing of exogenous firefly luciferase mRNA

Prior to carrier-free studies, we confirmed the biological activity of all siRNAs in HeLa and HT-29 cells after transfection with Lipofectamine reagent (Lipofectamine 2000™ in HeLa and Lipofectamine® LTX in HT-29). In both cell lines, anti-luciferase siRNAs achieved excellent dose-dependent knockdown of firefly luciferase after 8, 80 and 800 pM treatments (Figure 2). Consistently, siRNAs bearing internal modifications (propargyl or folic acid) showed much higher gene-silencing potency than the 3'-end modified siRNAs. As expected, scramble controls displayed no gene-silencing activity.

To assess the cellular uptake and delivery of siRNAs, HeLa and HT-29 cells were transfected following the respective carrier-free protocols described earlier with siRNA concentrations ranging from 1 to 3000 nM. In HeLa cells, gene-silencing activity was only observed in anti-luciferase siRNAs bearing folic acid modifications and not in the wild-

type (aL-wt) or propargyl siRNAs (aL-P1 to aL-P4) (Figure 3). As expected, scramble controls showed no gene-silencing activity, even with the folic acid modification present. The two siRNAs bearing centrally-placed folic acid modifications displayed the highest gene-silencing potency with IC₅₀ values of 171.0±48.8 and 128.95±9.7 nM for aL-F1 and aL-F2 respectively. Notably, at the lowest concentration tested, 1 nM, the centrally-modified folic acid siRNAs still showed ~20% gene-silencing activity. When the folic acid modification was placed at position 5 from sense strand 3' end (aL-F3), the gene-silencing potency was decreased by more than half (IC₅₀: 283.9±62.9 nM) whereas placing the folic acid modification at the 3' greatly reduced siRNA activity (IC₅₀: 1044±23.0 nM). To validate that the folic acid siRNAs are being internalized via FR, we subjected the same type of experiment to HeLa cells that were maintained in DMEM supplemented with folic acid. As seen in Figure 4, there is a significant decrease in the gene-silencing activity of centrally modified folic acid–siRNAs (aL-F1 and aL-F2) when free folic acid is present in the media. When the carrier-free siRNA transfection was performed in HT-29 cells, we observed no silencing activity for any of the tested siRNAs (Figure 5).

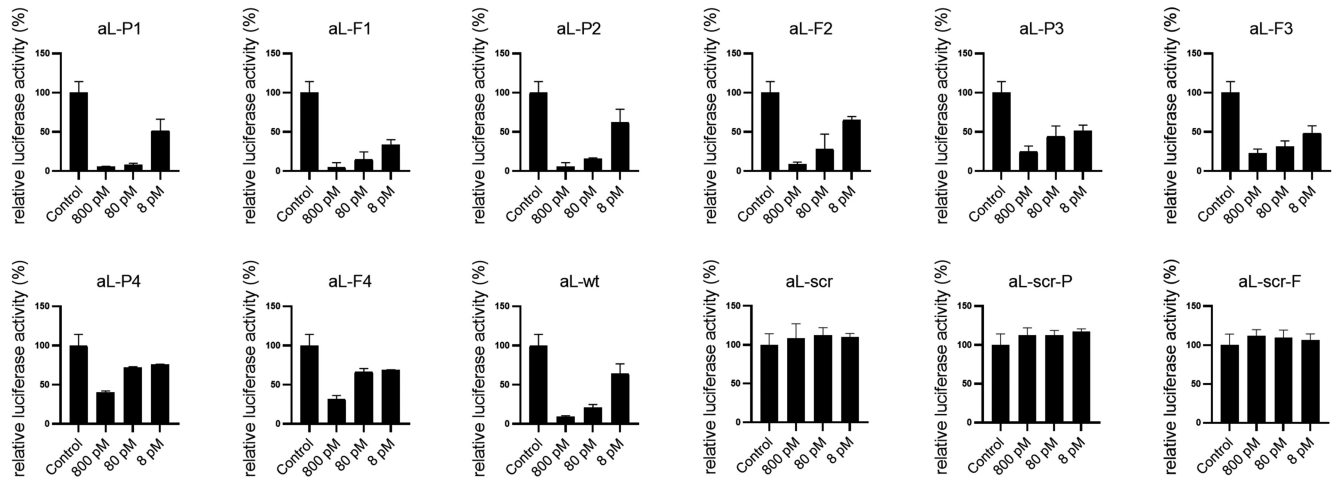
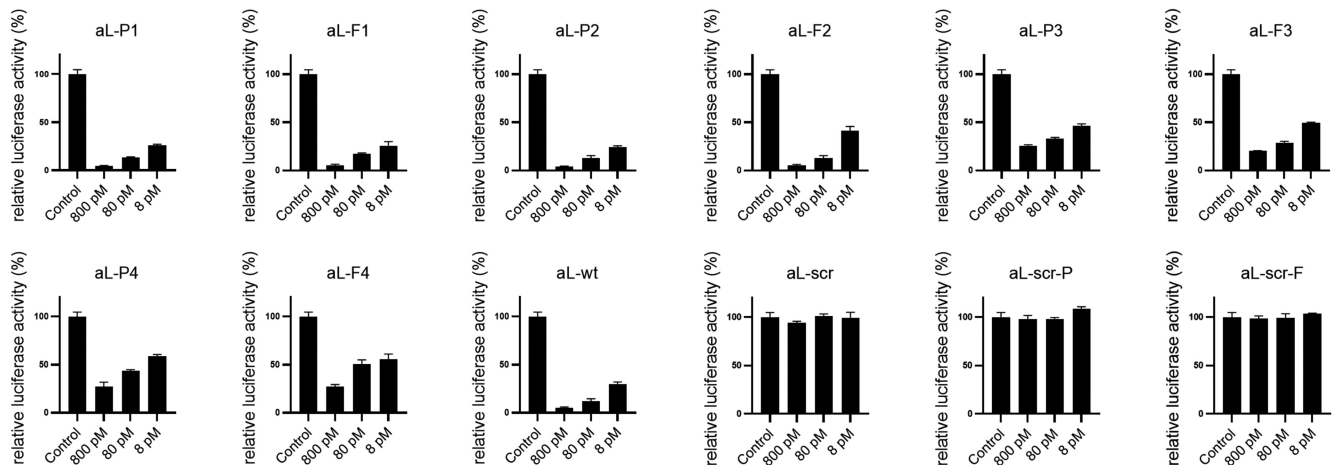
A HeLa**B HT-29**

Figure 2. Relative expression of firefly luciferase in HeLa (A) and HT-29 cells (B) 24 h after anti-luciferase siRNA transfections at 8, 80 and 800 pM using Lipofectamine. Firefly luciferase expression was assessed with a dual-luciferase reporter assay and was normalized to *Renilla* luciferase. Error bars indicate SD of at least two independent biological replicates.

Viability of HeLa and HT-29 cells after siRNA treatment

The XTT Cell Proliferation Assay was employed to assess HeLa and HT-29 cell viability after treatment with increasing siRNA concentrations (1, 25, 75, 150, 375, 750, 1500 and 3000 nM). At the highest concentration tested (3000 nM), cells treated with propargyl and folic acid–siRNAs displayed 80–90% viability, whereas cells treated with wild-type anti-firefly luciferase siRNA displayed reduced viability in HeLa cells (67%) (Supplementary Figure S4). At lower concentrations, cell viability remained high even after siRNA treatment.

Carrier-free gene silencing of endogenous Bcl-2 mRNA

The gene-silencing activity of internally-modified anti-Bcl-2 siRNAs was first tested in HeLa cells after transfection with Lipofectamine 2000TM. Both the propargyl and folic

acid-modified siRNAs (aB-P and aB-F, respectively) displayed ~70% knockdown after 20 nM treatment, comparable to wild-type siRNA (aB-wt), whereas scramble controls displayed no activity (Supplementary Figure S3). The carrier-free transfection protocol for this assay is described in the Supplementary Data file. In a carrier-free environment, the internally-modified folic acid–siRNA, aB-F, displayed potent gene-silencing activity of endogenous Bcl-2. At the highest concentration tested, 1 μ M, 70% knockdown was achieved (Figure 6).

DISCUSSION

Direct conjugation of folic acid to siRNAs has shown great success as a selective, self-delivering system to target cancer cells. Nevertheless, only 40–60% gene silencing has been achieved even after 1 μ M siRNA treatment (34). Therefore, given the promise of using folic acid as a delivery vehicle

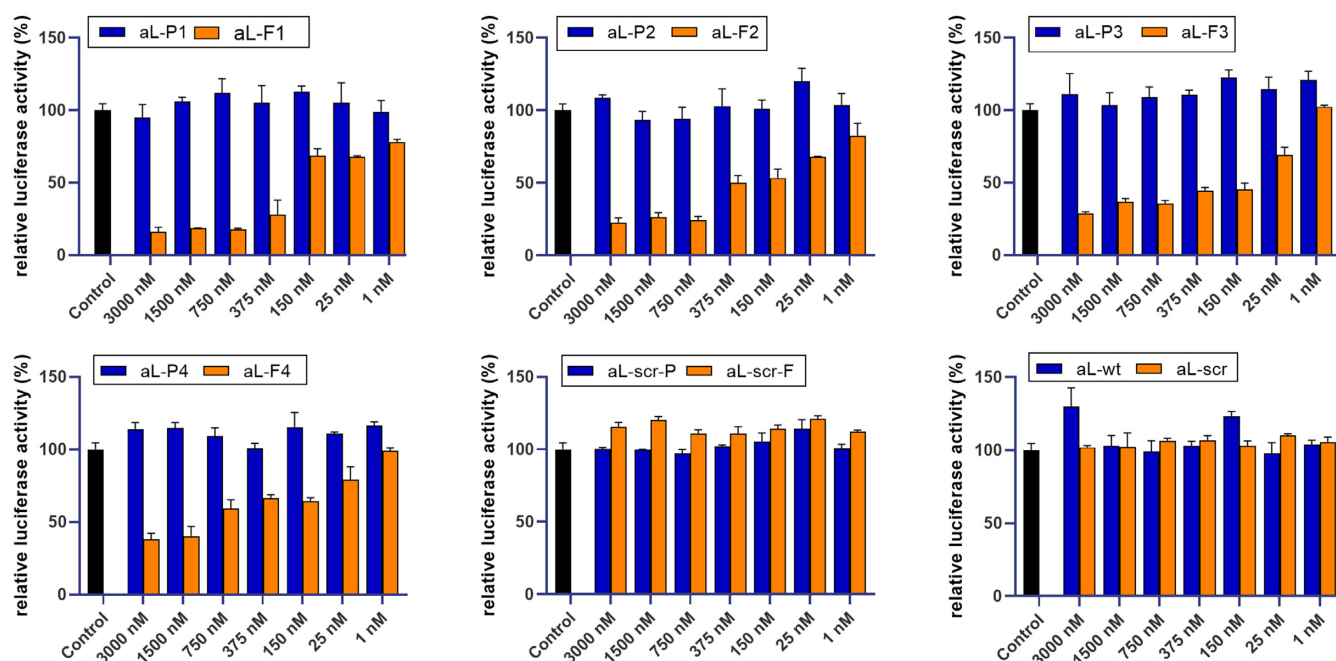


Figure 3. Relative expression of firefly luciferase in HeLa cells 16 h after anti-luciferase siRNA transfections at 1, 25, 150, 375, 750, 1500 and 3000 nM without the use of a transfection reagent. Firefly luciferase expression was assessed with a dual-luciferase reporter assay and was normalized to *Renilla* luciferase. Error bars indicate SD of at least two independent biological replicates.

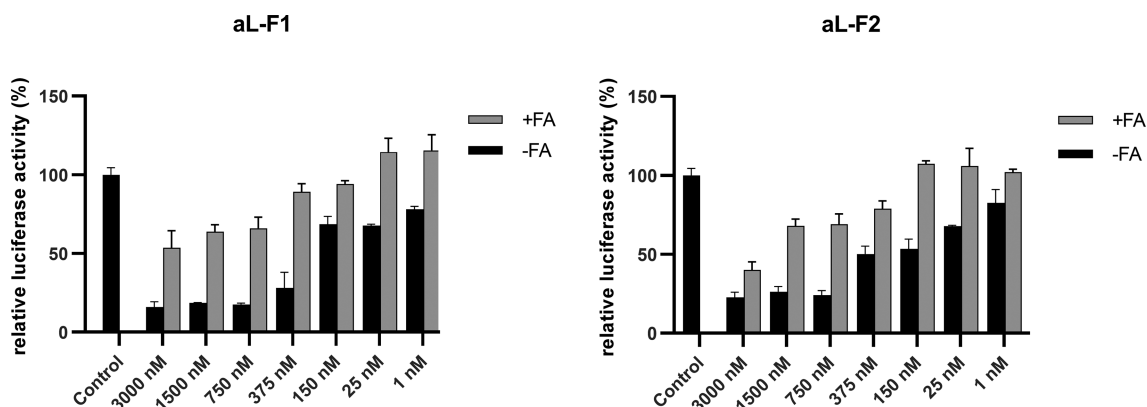


Figure 4. Relative expression of firefly luciferase in HeLa cells 16 h after carrier-free transfection of centrally-modified folic acid-siRNAs (aL-F1 and aL-F2). Cells were maintained in either folate-free RPMI 1640 (-FA) or folate-containing DMEM (+FA). Firefly luciferase expression was assessed with a dual-luciferase reporter assay and was normalized to *Renilla* luciferase. Error bars indicate SD of at least two independent biological replicates.

for siRNAs, there is room for improving its efficacy. Here we have investigated the gene-silencing activity of siRNAs bearing a triazole-linked folic acid modification at different positions within the sense strand, as previous work has only focused on the 3' and 5' ends. We have shown that placing the folic acid modification within the central region, spanning the Ago2 cleavage site of the sense strand, increased the gene-silencing activity of anti-luciferase and anti-Bcl-2 siRNAs.

We first assessed the biophysical properties of our synthesized siRNAs. Using CD spectroscopy, we confirmed that our siRNA duplexes adopted an A-form alpha helix conformation (Supplementary Figure S1). RISC recognizes the A-form major groove of the siRNA helix, so the ability of modified siRNAs to adopt an A-form helical structure is de-

sirable for proper RNAi activity (40). We then assessed the thermal stability of each siRNA duplex, as the thermodynamic properties of siRNA have been shown to play a role in their silencing activity (41). Modifications placed at or close to the 3' end did not cause significant thermal destabilization. This was expected, as this area has been shown to be fairly tolerant to chemical modifications (14). On the other hand, modifications spanning the central region of the sense strand caused significant thermal destabilization. Some studies suggest that destabilization in this region can lead to increase silencing activity (39,42) and previous work from our group has reported success using internally-modified siRNAs bearing a variety of chemically-modified spacer linkages (36,43). A crucial step for RNAi function is the dissociation of the sense strand, facilitated by Ago2

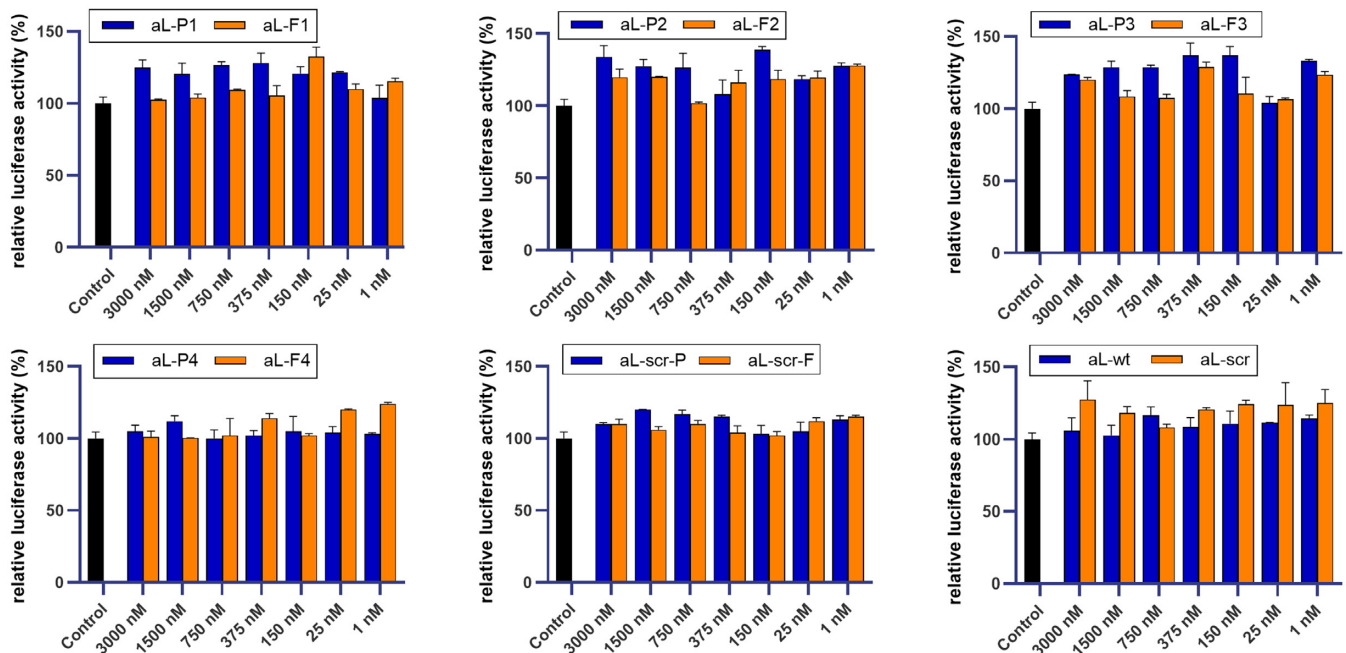


Figure 5. Relative expression of firefly luciferase in HT-29 cells 16 h after anti-luciferase siRNA transfections at 1, 25, 150, 375, 750, 1500 and 3000 nM without the use of a transfection reagent. Firefly luciferase expression was assessed with a dual-luciferase reporter assay and was normalized to *Renilla* luciferase. Error bars indicate SD of at least two independent biological replicates.

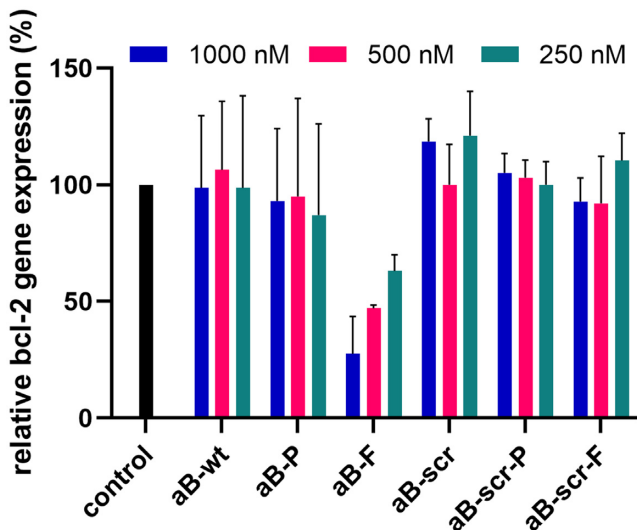


Figure 6. Normalized Bcl-2 gene expression in HeLa cells 24 h after carrier-free transfection with internally-modified anti-Bcl-2 and scramble siRNAs at 250, 500, 1000 nM concentrations. Data was normalized using the 18S gene as a reference gene. Error bars indicate SD of two independent biological replicates.

cleavage at the central region. It has been proposed that low thermal stability in this region could improve RNAi activity by facilitating passenger strand release (44). To investigate the gene-silencing potency of centrally-modified folic acid-siRNAs, we first targeted the exogenous gene firefly luciferase in two cell lines, HeLa and HT-29. HeLa cells are derived from human cervical cancer and HT-29 cells are derived from human colon cancer. We assessed the relative ex-

pression of FRs in HeLa and HT-29 cells using flow cytometry and found that HeLa cells displayed a 3-fold increase in FR expression compared to HT-29 (Figure 1). Although this is not a quantitative measure, a 3-fold increase in receptor expression can be biologically significant. Multiple examples are provided in Leamon's study (26), which quantitatively measured the expression of FR in various human cancer and normal tissues. High FR-positive tissues and cells, such as HeLa, express at least 6 pmol FR/mg protein whereas tissues or cells expressing no more than 2.5 pmol FR/mg protein are considered to have low FR expression, suggesting that a small difference in expression can lead to significantly different biological activity. Several literature reports indicate that HeLa cells express high levels of FR (45) whereas HT-29 cells express low levels of FR (46). Based on this, HeLa was chosen as the FR-positive cell line and HT-29 as the FR-negative cell line.

In HeLa cells, we show that internally-modified propargyl and folic acid-siRNAs displayed more potent gene-silencing activity than their 3'-modified counterparts after transfection with Lipofectamine 2000™ (Figure 2A). Even in the absence of a transfection reagent, internally-modified folic acid-siRNAs aL-F1 and aL-F2 still displayed enhanced gene-silencing potency and much lower IC₅₀ values than aL-F3 (modified at position 5 from sense strand 3' end) and aL-F4 (modified at the 3' end). We only observed 40–65% knockdown after 0.75 μM treatment of aL-F3 and aL-F4. However, treatment with the centrally-modified folic acid siRNAs aL-F1 and aL-F2 at the same 0.75 μM concentration led to 80% knockdown (Figure 3), a significant improvement from literature reports. Although Low's study reported selective *in vivo* delivery of 5'-modified folic acid-siRNAs to tumours in mice bearing KB tumour xenografts,

these siRNAs were trapped in intracellular endosomes after internalization and did not display efficient gene-silencing activity (35). Carell's study, on the other hand, achieved moderate gene-silencing activity with a 3'-modified folic acid siRNA (34). This study targeted exogenous luciferase mRNA in HeLa cells and reported ~50% gene-silencing activity after 1 μ M siRNA treatment. One potential reason for this saturation could be due to off-target effects, namely, the strand selection process. If the 3' folic acid-modified passenger strand is selected as the guide strand for the RISC complex, it is possible that reduced overall gene silencing may occur. The central region of the antisense strand has been shown to be less tolerant to chemical modifications (47). Therefore, by using the central region in the passenger strand for a folic acid modification, it is possible that enhanced efficacy could be attributed to loss of passenger strand uptake by the RISC complex.

To validate that the folic-acid siRNAs are being internalized via FRs, we performed a folic acid competition study. Following the same carrier-free protocol described earlier, we transfected the two centrally-modified folic acid-siRNAs (aL-F1 and aL-F2) into HeLa cells that were maintained in DMEM supplemented with folic acid (9 μ M). FRs are found on the cell surface and are able to internalize folic acid and folic acid-conjugates via receptor-mediated endocytosis. When excess folic acid was present in the media, there was a significant decrease in siRNA gene-silencing activity compared to previously-described studies in folate-free media (Figure 4). After confirming the self-delivering properties of our anti-luciferase folic acid-siRNAs in HeLa cells, we investigated their selectivity for FR-expressing cell lines by testing them in FR-negative HT-29 cells. We first performed the transfection using Lipofectamine[®] LTX to ensure that the siRNAs were biologically active once inside the cell. We observed a similar pattern of gene-silencing activity as we did in HeLa cells, with internally-modified siRNAs displaying much higher potency than 3'-modified siRNAs (Figure 2B). In the absence of a transfection carrier, however, none of the tested siRNAs displayed activity, confirming their selectivity for FR-expressing cells (Figure 5). In both cell lines, siRNA treatment caused low to no cytotoxic effects (Supplementary Figure S4), even at the highest concentrations tested (3000 nM). Given the selectivity and potent gene-silencing activity of our internally-modified folic acid-siRNAs against the exogenous target firefly luciferase, we designed siRNAs targeting the endogenous gene Bcl-2. This oncogene is overexpressed in 50–70% of all human cancers and is a desirable target for siRNA therapeutics (48–50). The triazole-linked folic acid modification was incorporated at position 10 from the sense strand 5' end of our anti-Bcl-2 siRNA (aB-F) and gene-silencing activity was assessed using real-time polymerase chain reaction (RT-PCR) in HeLa cells, which endogenously express *bcl-2*. This internally-modified folic acid-siRNA displayed potent gene-silencing activity even in the absence of a transfection reagent (Figure 6). Notably, we observed ~72% knockdown of endogenous Bcl-2 after 1 μ M siRNA treatment.

In summary, we report a straightforward and efficient post-column CuAAC synthetic strategy to prepare self-delivering folic acid-siRNAs that selectively target FR-

expressing cells. Furthermore, we have developed an approach to enhance the gene-silencing potency of folic acid-siRNA constructs by modifying the central region of the siRNA sense strand and achieved improvement in siRNA activity compared to literature reports. Overall, our data show that siRNAs with internal folic acid modifications are able to effectively downregulate the expression of both exogenous and endogenous gene targets with minimal toxicity. Given that folate receptors are vastly overexpressed in a variety of cancers, our synthetic approach could be employed to achieve selective delivery of siRNAs to cancer cells without the use of transfection reagents or sophisticated carriers while maintaining potent RNAi activity. Next steps could involve examining our folic acid-siRNA conjugates in higher-level organisms that have folic receptor alpha positive tumors. Therefore, our work can open new avenues for the design and development of novel RNAi-based cancer therapeutics.

DATA AVAILABILITY

Data available in the supplementary material.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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