Supporting Information

Minor-groove-modulating adenosine replacements control protein binding and RNAi activity in siRNAs

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EXPERIMENTAL DETAILS

Synthesis of RNAs:
RNA oligonucleotides were synthesized on an ABI 394 synthesizer (DNA/Peptide Core Facility, University of Utah) using 5'-DMTrO, 2'-OTBDMS protected β-cyanoethyl phosphoramidites. 5'-Biotin modifications were installed with a BiotinTEG phosphoramidite (Glen Research). The preparation of the N^2-propargyl-2-aminopurine phoshoramidite was reported previously(*1*). RNAs were deprotected, gel-purified, quantified and analyzed by ESI-MS as previously described(*1*).
List of calculated and observed zero-charge mass values for propargyl-containing RNAs (P=passenger, G=guide): P3+16 Propargyl: Calc. 6876.05, Obs. 6876.03; P9+14 Propargyl: Cacl. 6876.05, Obs. 6876.05; G2+14 Propargyl: Cacl. 6554.84, Obs. 6554.85; G2 Propargyl: Cacl. 6516.82, Obs. 6516.80; G14 Propargyl: Cacl. 6516.82, Obs. 6516.82.

CuAAC reaction on RNAs:
A solution of 10 nmol propargyl-containing RNA in 1 uL H_2O was treated sequentially with tris-[1-(3-hydroxypropyl)-1H-[1,2,3]triazol-4-yl)methyl]amine (THPTA) ligand(*2*) (1 uL, 1 M in H_2O), $CuSO_4$ (1 uL, 100 mM in H_2O), sodium ascorbate (1 uL, 1 M in H_2O) and the corresponding azide (1 uL, 0.5 M N-azidoacetyl-D-mannosamine(*3*) in H_2O or 50 mM 1-(2-azidoethyl)-piperidine(*4*) in 0.5 M Tris-HCl, pH 8.0). The resulting solution was incubated for 4 h at room temperature. The reaction mixture was diluted to 2x the original volume with PAGE loading buffer (80% formamide containing 10 mM EDTA). The RNA was gel-purified and quantified (as above). Lypholization gave white pellets, which were fully soluble in H_2O. In all cases the gel contained a single band which migrated slower than the propargyl-containing RNAs, confirming complete conversion and purity of the triazole-containing products. ESI-MS analysis confirmed the identity of the click products.
List of calculated and observed zero-charge mass values for triazole-containing RNAs (P=passenger, G=guide): P3+16 PipNEt: Calc. 7184.30, Obs. 7184.27; P9+14 PipNEt: Calc. 7184.30, Obs. 7184.22; G2+14 PipNEt: Calc. 6863.08, Obs. 6863.07; G2 PipNEt: Calc. 6670.94, Obs. 6670.90; G14 PipNEt: Calc. 6670.94, Obs. 6670.98; P3+16 ManNAc: Calc. 7400.24, Obs. 7400.23; P9+14 ManNAc: Calc. 7400.24, Obs. 7400.28; G2+14 ManNAc: Calc. 7079.02, Obs. 7078.86; G2 ManNAc: Calc. 6778.91, Obs. 6778.88; G14 ManNAc: Calc. 6778.91, Obs. 6778.88.

Thermal analysis:
The thermal stability of the piperidine ethyl triazole modification was analyzed in a 12mer RNA duplex as previously described(*1*).

siRNA duplex formation:
Hybridization to form siRNA duplexes was accomplished by combining equal amounts of purified passenger and guide strands to a final concentration of 1 uM in 10 mM Tris-HCl, 50 mM KCl, pH 7.5. The samples were heated at 95 °C for 5 min followed by cooling to room temperature over a period of approximately 2 h.

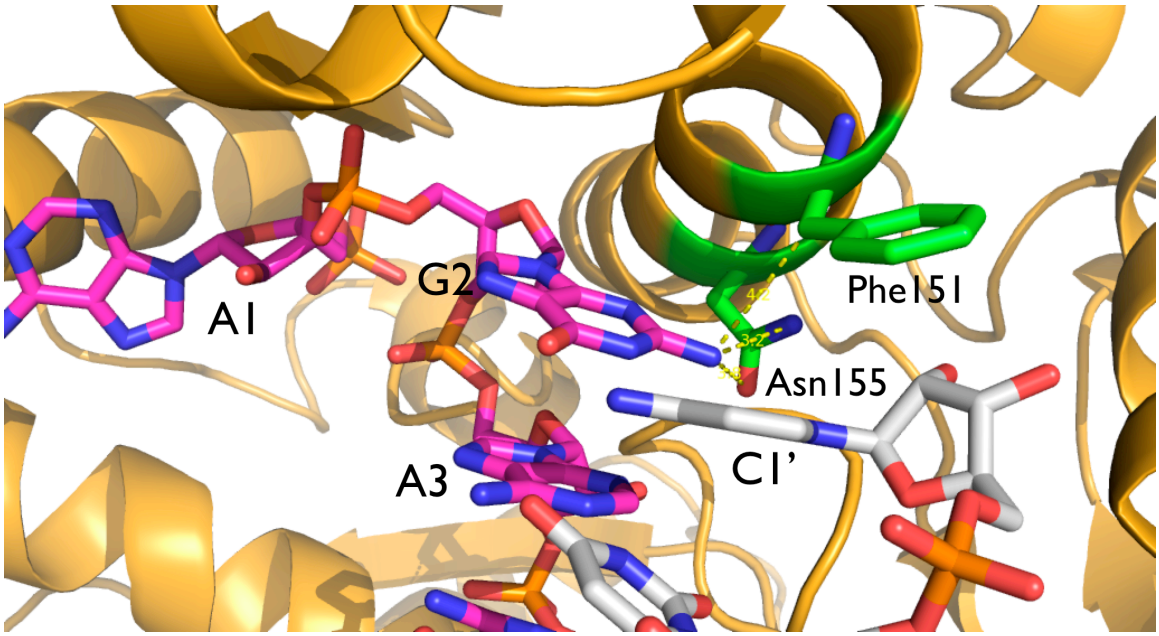
Cell culture:
HeLa cells (ATCC) and U87 cells (ATCC) were grown at 37 °C in humidified 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 50 ug mL^{-1} gentamycin. The cells were maintained in exponential growth.

Transfection and RNAi activity assay:
HeLa cells were reverse-transfected using siPORT NeoFX transfection reagent (Ambion) according to the manufacturer's instructions. Briefly, cells grown in flasks at approximately 80% confluence were detached with accutase (Innovative Cell Technologies, Inc.) and diluted in fresh medium (DMEM, 10% FBS) without antibiotics to a concentration of 1 x 10^5 cells mL^{-1}. A vector was prepared from the psiCHECK2 plasmid (Promega) that contained the reporter genes renilla and firefly luciferase (hRluc and hluc+, respectively) with the caspase 2 siRNA sequence inserted into the 3' untranslated region of the former (psiCHECK2-Cas2). Renilla luciferase was used as a reporter and firefly luciferase was used as control. Cotransfections of the plasmid and siRNAs were performed with siPORT NeoFX in 96 well plates. Each well contained 0.5 µL siPORT, 50 ng psiCHECK2-Cas2, and .01-10 uM siRNA (hybridization buffer for negative controls, see section, "siRNA Duplex Formation") in 17.5 µL Opti-Mem (Gibco) for a total volume of 20 µL. This was overlaid with 80 µL of the cell suspension described above for a total of 8000 cells per well. The cells were harvested 21 hours post-transfection and lysed with 1x passive lysis buffer (Promega). The lysates were analyzed using the Dual Luciferase Assay Reporter system (Promega) according to the manufacturer's instructions. Luciferase activity was measured with a Victor 3 multilabel plate reader (Perkin Elmer), using a delay time of 2 s and an integrate time of 10 s. RNAi activity was measured as the normalized ratio between hRluc and hluc+, and reported as the average of three independent experiments.

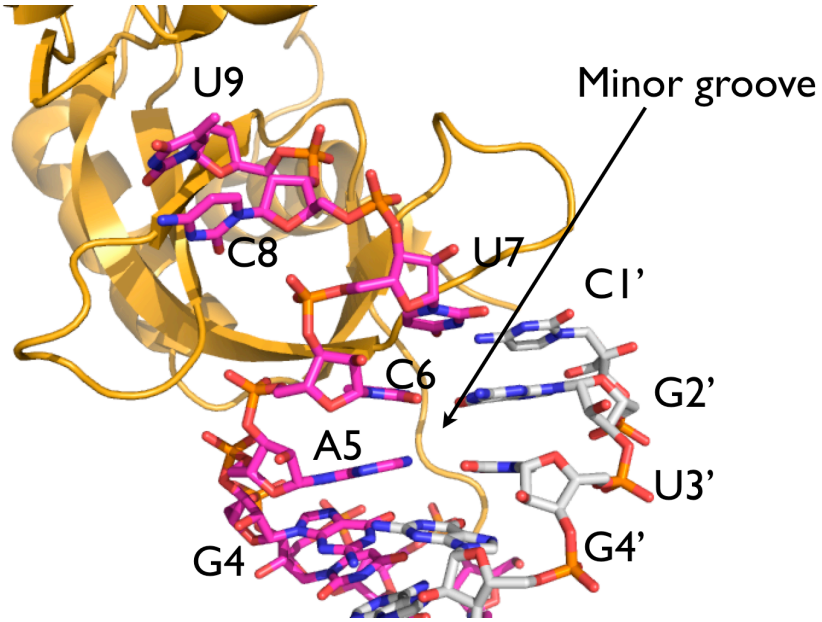
siRNA protein binding assay:
21 h before lysis, U87 cells (approximately 8 x 10^6 in a 75 cm^2 flask) were treated with human interferon-α A (PBL Interferon Source) to a final concentration of 1 x 10^6 U L^{-1}. The interferon-treated cells were washed twice with PBS and lysed by shaking with 3 mL solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, supplemented with protease inhibitor cocktail (ProteoBlock, Fermentas)) for 30 min on ice. The lysates were clarified by centrifugation at 14000 x g at 4 °C for 20 min and used directly in pull-down experiments. Magentic steptavidin beads (0.5 mg, 50 uL) (Dynabeads M-280, Invitrogen) were prepared for RNA manipulation according to the manufacturer's protocol, re-

suspended in 350 uL binding buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl) and coated with 100 pmol of siRNA (100 uL) by gently rocking for 30 min at room temperature. The siRNA-coated beads were washed twice with binding buffer (500 uL) and once with solubilization buffer (500 uL) and incubated with 0.5 mL cell lysate for 20 min at room temperature. Beads without siRNA were also incubated with cell lysate and served as a control. After incubation, the beads were washed four times with 500 uL wash buffer (20 mM HEPES, pH 7.9, 2.5 mM MgCl2, 100 mM KCl, 20% glycerol, 0.5 mL DTT, 0.2 mg/mL yeast RNA, 0.2 mg/mL salmon sperm DNA). Proteins were eluted from the beads by heating in loading buffer, separated on 6% SDS-PAGE and transferred to a PVDF membrane for Western blotting. Membranes were blocked with blotting-grade milk (Bio-Rad), incubated with ADAR1 antibody (Santa Cruz Biotechnology, 1 : 5000 dilution), washed with TBS-Tween, and incubated with alkaline phosphotase-conjugated secondary antibody (Santa Cruz Biotechnology, 1 : 2000 dilution). The proteins were detected using ECF substrate (GE Healthcare) on a Typhoon Trio Variable Mode Imager (GE Healthcare) and band intensities were quantified using ImageQuant software (Molecular Dynamics). The membrane was stripped (Glycine-HCl, pH 2.2, 1% Tween 20, 0.1% SDS) and reprobed with PKR antibody (Santa Cruz Biotechnology, 1 : 1000 dilution). ADAR1 and PKR binding affinity is reported as the average ratio of band intensities of modified siRNA to native siRNA for three independent experiments, unless specified otherwise.

SUPPLEMENTARY FIGURES



Supplementary Figure 1: Crystal structure of a Piwi protein from *Archaeoglobus fulgidus* bound to an siRNA-like RNA(*5*) (PDB code: 2BGG). The strand bound by its 5'-end (shown in purple), which mimics the 5'-end of the guide strand of an siRNA bound to mRNA target, is in close contact with amino acids residues in the minor groove at position 2 (guanosine).



Supplementary Figure 2: Crystal structure of the PAZ domain from human Ago1 in complex with an siRNA-like duplex(*6*) (PDB code: 1SI2). The minor groove is free of protein contacts towards the duplex end.

References:

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