Site-specific conjugation of drug-like fragments to an antimiR scaffold as a strategy to target miRNAs inside RISC

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Materials and instruments:

Chemicals were purchased from Acros, Aldrich and TCI. Phosphoramidites were purchased from Thermo Fisher. The activator 5-(Benzylthio)-1H-tetrazole (BTT) was purchased from Biosolve. All oligonucleotides used in this work were synthesized with the MM12 synthesizer (Bio Automation Inc.) on 1000 Å UnyLinker CPG (ChemGenes). The coupling time of phosphoramidites was set to 2 x 90 s. The oligonucleotides were purified on an Agilent 1200 series preparative HPLC on a Waters XBridge OST C-18 column (10 X 50 mm, 2.5 µm) at 65 °C. Solvent A: 0.1 N aqueous triethylamine/acetic acid, pH 8.0; solvent B: 100 % MeOH; flow-rate: 5 mL/min. Gradient for DMT-on purification: 5 % to 80 % solvent B over 8 min. Gradient for DMT-off purification: 1 % to 60 % solvent B over 12 min; flow-rate: 5 mL/min. Fractions containing the product were collected and dried in a miVac duo SpeedVac (Genevac). The oligonucleotides were analyzed by LC-MS (Agilent 1200/6130 system) on a Waters Acquity OST C-18 column, 2.1 X 50 mm, 1.7 μ M, 65 °C. Buffer A: 0.4 M HFIP, 15 mM triethylamine; buffer B: MeOH. Gradient: 10-80 % B in 6 min; flow-rate: 0.3 mL/min. UVabsorption of the final products was measured in triplicate on a nanodrop 2000 spectrophotometer (Fisher Scientific). Concentrations were calculated based on the nearest neighbor model with an inhouse programmed software. The extinction coefficients of the oligonucleotide-small molecule conjugates were corrected by the extinction coefficients of the appended small molecules (table S3). Thin layer chromatography was done on silica gel 60 Å F₂₅₄ aluminium sheets (Merck). Preparative column chromatography was carried out on silica gel, 60 Å (Fluka) using the Isolera Four flash chromatography system (Biotage). Chemical shifts are given in ppm, J values are given in hertz (Hz), NMR spectra were referenced to the residual undeuterated solvent.

Thermal denaturation measurements

Oligonucleotide concentrations were determined by measuring the absorbance on a Cary 300 Bio UV spectrometer (Varian) at 260 nm and dividing the obtained values by the calculated extinction coefficients according to the nearest neighbour method.¹ Melting curves were measured on a Cary 300 Bio UV spectrometer (Varian) equipped with a thermoelectrical controller using a strand concentration of 2 μ M in 5 mM Na₂HPO₄ (2.5 mM for **3**-series), 5 mM NaH₂PO₄•H₂O, 100 mM NaCl and 0.1 mM Na₂EDTA•2H₂O buffer, pH 7 (pH 6.4 for **3**-series). The change in absorbance at 260 nm as a function of time was recorded while the temperature was decreased/increased at a cooling/heating rate of 0.5 °C min⁻¹. *T*_m was determined by taking the local maximum of the first derivatives of the absorbance vs. temperature curve. All melting curve measurements were performed in triplicates.



Figure S1: Melting temperatures (T_m 's) of: nalidixic acid antimiR conjugates **3j**, **4j**, **5j** and **6j** (left); antimiR conjugates with fragments **b**-i conjugated to antimir at the 3-position **3b**-i (right). T_m 's were calculated using the first derivative method. Values are average of 3 technical measurements. Error bars, SD (n=3). The T_m of SPC3649 is reported as 80 °C (ref 19 in the main manuscript).

Luciferase assays

An artificial miR-122-5p target sequence (AAACTCGAGCCGCAGTGCCCCACCCAGTCCGCCAA<u>CAAA</u>-<u>CACCATTGTCACACTCCA</u>CCCCCTCCAGCTCCAGTTTCCAAGCGGCCGCAAA) was cloned into the psiCHECK-2 vector (C8021, Promega, Fitchburg) according to the manufacturer's protocol.

Huh-7 cells were seeded at 7500 cells/well in opaque white 96-well plates (136101, Nunc, Roskilde) in 80 µl DMEM Glutamax (31966-021, Gibco, Life Technologies, Carlsbad) supported with 10% FBS. Transfections were performed after 6 h (antimiRs, 0.6-40 nM, Lipofectamine 2000 (11668-019, Invitrogen, Life Technologies, Carlsbad) and after further 18 h (psiCHECK-2 miR-122-5p-reporter, 20 ng, JetPEI (101-10, Polyplus transfections, Illkirch) according to the manufacturer's protocol. The dual luciferase assay was performed after further 48 h with the Dual-Glo Luciferase Assay System (E2980, Promega, Fitchburg) on a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad). Renilla counts were normalized on firefly counts for each well and average values and SD of triplicates were calculated. 2-way ANOVA analysis was used to evaluate treatment effects. If ANOVA was significant, Bonferroni's post test was performed comparing to the lowest dose. P values: * P < 0.05; ** P < 0.01; *** P < 0.001.

Quantification of miR-122 antimiRs by chemical-ligation qPCR (CL-qPCR) in cell lysates and after AGO2 RNA immunoprecipitation (RIP)

Huh-7 cells were plated in 15-cm culture dishes $(4 \times 10^6$ live cells/dish) and 24 h later transfected with antimiRs **1**, **3a**, and **5a** at 100 nM final concentration. 25 H post-transfection, cells were washed once with 10 ml PBS, placed on ice and irradiated twice at 254 nm with 100 mJ (CL-1000 UV-Crosslinker, UVP). After irradiation, cells were scraped in 5 ml ice-cold PBS, pelleted at 500g at 4 °C for 5 min and lysed in 1.5 ml of NP40 lysis buffer for 15 min on ice. The lysate was cleared at 14,000g at 4 °C for 15 min, 3-5% of total volume was snap-frozen and the rest was incubated with the AGO2 (clone 9A11; Ascenion, Munich, Germany) antibody-coupled Protein G Dynabeads (Invitrogen, 100-03D) for 1 h at 4 °C with gentle rolling. The anti-AGO2 RNA immunoprecipitation (RIP) and the subsequent RNA isolation were performed as previously described.² Total RNA was isolated from lysates (3-5% of total volume) to quantify cellular uptake of the antimiRs, and from anti-AGO2 RIPs in order to quantify their uptake in AGO2. The antimiRs were detected by chemical-ligation qPCR (CL-qPCR) as previously described (Scheme S1).³ The CL-qPCR was adapted for detection of miR-122 antimiRs (Table S1). The chemical-ligation reactions were incubated at 33 °C for 2 h. The qPCR reaction was performed in a Light Cycler 480 system (Roche Diagnostics AG, Switzerland) and consisted of an activation cycle at 95 °C for 10 min and 50 cycles with following cycling conditions: 3 s/95 °C, 30 s/48 °C, 10 s/72 °C and a ramping speed of 3 °C/s. AntimiRs were serially diluted ranging from 10 ng/µl to 10 fg/µl in 10 ng/µl Poly(A) (GE Healthcare, #27-411-01) and the resultant standard curves were used for the absolute quantification of antimiRs in cell lysate and after AGO2 RIP (Figures S2-4). The No Template Control (NTC) containing only 10 ng/µl Poly(A) was used to determine the background levels of the qPCR primers. Unpaired t test was used to compare the quantifications of **1** and **3a**. P values: ** P < 0.01.



Scheme S1: The mechanism of the chemical-ligation qPCR (CL-qPCR). Chemically modified antimiR acts as template for the two DNA ligators (A). Upon hybridization to the antimiR, the ligation reaction (B) occurs leaving a modified DNA ligation product that is detected by the following qPCR method. Scheme adapted from ³.

Sequence ID	Sequence (5'-> 3')	MS calc.	MS found
5'-Ligator	TTAAACCATGGAGTGT-PS ^a	5015.80	5014.71
3'-Ligator	BPS-GACAATGGAACCAGT ^b	4826.14	4896.00
Reverse primer	ACTGGTTCCATTGTCAC	-	-
Forward primer	C^{Cy3}TCCCTCCCTCGATTTAAACCATGGAGTGT ^d	9708.30	9708.07
Anti-primer	AAATCGAGGGAGGG A^{BHQ}G^e	5599.65	5598.15

3'-thiophosphate DNA ligator was synthesized following the previously described protocols (see Materials and instruments) with the exception that: (1) the 3'-phosphate CPG (Glen Research, #20-2900-01) was used and (2) the oligonucleotide deprotection and cleavage from the solid support was carried out in a conc. NH₄OH (25%) solution for 15 h at room temperature. A detailed procedure can be found in ref³.

^b BPS: 5'-o-biphenylsulphonyl-2'-deoxynucleoside; the phosphoramidite was kindly provided by Iwan Beuvink (NIBR, Novartis Pharma AG, Basel, Switzerland). 5'-BPS DNA ligator was prepared following the previously described protocols (see Materials and instruments) with the exception that: (1) the Standard Support-dT 2000 Å was used as CPG instead of UnyLinker 500 Å, (2) PAC-dA phosphoramidite was used instead of the regular benzoyl protected phosphoramidite and (3) the oligonucleotide deprotection and cleavage from the solid support was carried out under especially mild conditions (conc. NH₄OH (25%) solution; 4 h; room temperature) to ensure the integrity of the BPS leaving group on the terminal position. The measured mass of the final pure product shows that the isobutyryl protecting group on the modified G is not removed by the mild deprotection conditions. In time-course studies with similar sequences (data not shown) we (J. Hunziker) have noted that this protecting group is resistant to mild ammonia treatment, however use of the standard concentration curves to quantify the antimiRs ensures that it does not affect the qPCR measurements and subsequent calculations. A detailed procedure can be found in ref³.

^c from Microsynth AG, Balgach, Switzerland.

^d Cy3-label introduced as described in ref. ⁴ using a 2'-*O*-propargyl-cytidine phosphoramidite. ^e BHQ-label introduced as described in ref. ⁴; making use of a phosphoramidite described in ref. ².







Figure S2: Standard concentration curves obtained for the absolute quantification of miR-122 antimiR **1** in cell lysate (left) and after AGO2 RNA immunoprecipitation (RIP) (right). AntimiR **1** was serially diluted in 10 ng/ μ l Poly(A). Signals that did not fall within the linear range of the standard curve are not shown. Values are average of 3 technical measurements. Error bars, SD (n=3). Two independent biological replicates are shown.

Experiment 1



Experiment 2



Figure S3: Standard concentration curves obtained for the absolute quantification of miR-122 antimiR **3a** in cell lysate (left) and after AGO2 RNA immunoprecipitation (RIP) (right). AntimiR **3a** was serially diluted in 10 ng/ μ l Poly(A). Signals that did not fall within the linear range of the standard curve are not shown. Values are average of 3 technical measurements. Error bars, SD (n=3). Two independent biological replicates are shown.

Experiment 1



Experiment 2



Figure S4: Standard concentration curves obtained for the absolute quantification of miR-122 antimiR **5a** in cell lysate (left) and after AGO2 RNA immunoprecipitation (RIP) (right). AntimiR **5a** was serially diluted in 10 ng/ μ l Poly(A). Signals that did not fall within the linear range of the standard curve are not shown. Values are average of 3 technical measurements. Error bars, SD (n=3). Two independent biological replicates are shown. n.d., not detected.

Synthesis and characterization of 2'-pent-4-yn-1-yl-adenosine phosphoramidate 2⁵



Scheme S2: Synthesis of the 2'-pent-4-yn-1-yladenosine phosphoramidite. Reagents and conditions: a) 5-Chloro-1-pentyne, NaH, dry DMF, TBAI, 65 °C, argon, 48 h; b) DMF-DMA, DMF, 50 °C, 1 h; c) DMTr-chloride, cat. DMAP, dry pyridine, 12 h, 0 °C to rt, argon; d) 2-cyanoethyl diisopropylphosphoramidochloridite, dry CH_2Cl_2 , DIPEA, rt, 30 min, argon.

(2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-(hydroxymethyl)-4-(pent-4-yn-1-yloxy)tetrahydrofuran-3-ol (9)



Adenosine (5 g, 18.5 mmol) was dried *in vacuo* and dissolved in 400 mL of dry DMF under argon by heating the solution shortly to 100°C. Then it was cooled to 0 °C (ice bath). NaH was added (2 g, 20 mmol) and the turbid mixture was stirred for 1 h at 0 °C. 5-Chloro-1-pentyne (2.5 g, 23 mmol) was added dropwise, followed by TBAI (3 g,

8.1 mmol) and the mixture was heated to 65 °C and stirred for 48 h at that temperature. Then, the volatiles were removed by rotary evaporation. The residue was taken up in MeOH (20 mL) and the precipitated inorganic salts were filtered off. Then, the product (1.9 g, 31 %) was purified by flash chromatography using a gradient of CH₂Cl₂/MeOH 100:0 to 80:20. δ H (400 MHz; DMSO-d₆) 8.38 (1H, s, H-2), 8.15 (1H, s, H-8), 7.33 (2H, s, 6-NH₂), 6.00 (1H, d, ³J = 6.08 Hz, H-1'), 5.39 (1H, dd, ³J = 4.76 Hz and ³J = 6.92 Hz, 5'-OH), 5.18 (1H, d, ³J = 5.24 Hz, 3'-OH), 4.47 (1H, dd, ³J = 5.84 Hz and ³J = 5.04 Hz, H-2'), 4.31 (1H, ddd, ³J = 4.92 Hz and ³J = 4.68 Hz and ³J = 3.28 Hz, H-3'), 3.98 (1H, dd, ³J = 6.84 Hz and ³J = 3.48 Hz, H-4'), 3.71-3.43 (4H, m, 2 x H-5' and 2 x H, 2'-O-CH₂), 2.66 (1H, t, ⁴J = 2.64 Hz, CH), 2.10 (2H, dt, ³J = 6.84 Hz and ⁴J = 2.48 Hz, 2'-O-CH₂-CH₂), 1.61 (2H, tt, ³J = 6.60 Hz and ³J = 6.84 Hz, 2'-O-CH₂-CH₂). δ C (100 MHz, DMSO-d₆) 156.5 (C-6), 156.5 (C-2), 148.8 (C-4), 139.6 (C-8), 117.8 (C-5), 86.3 (C-1'), 86.0 (C-2'), 80.9 (C-4'), 85.8 (C=CH), 72.5 (C=CH), 71.1 (C-3'), 69.0 (2'-O-CH₂), 61.2 (C-5'), 28.1 (2'-O-CH₂-CH₂), 14.2 (2'-O-CH₂-CH₂). ESI-MS: positive mode 333.79 ([M+H]⁺). Calc.: 333.34.

N'-(9-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-Methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxy-3-(pent-4-yn-1yloxy)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)-*N*,*N*-dimethylformimidamide (10)



A solution of the 2'-O-pent-4-yn-1-yladenosine **9** (1.0 g, 3.0 mmol) in DMFdimethylacetale (3 mL) and dry DMF (10 mL) was stirred for 1 h at 50 °C under an atmosphere of argon. The volatiles were removed by rotary evaporation, the residue was dried in vacuum and directly used in the next step. To a solution of the N6-DMF-protected 2'-O-pent-4-yn-1-yladenosine (1.18 g, 3.0 mmol) in dry pyridine (10 mL) were added DMAP (0.033 g, 0.3 mmol), and 4,4'-dimethoxytriphenylmethyl chloride (1.12 g, 3.3 mmol) at 0 °C (ice bath) under an atmosphere of argon. The reaction was stirred overnight allowing it to warm up to 25 °C. Then, it was cooled again to 0 °C and 1 mL of methanol was added to quench the reaction for 30 min. The volatiles were removed by rotary evaporation and the residue was dissolved in dichloromethane (100 mL). The organic layer was washed with ice-cold brine (3 x 50 mL), ice-cold water (1 x 50 mL), dried over Na_2SO_4 , and removed by rotary evaporation. The product (2.8 g, 81 %) was purified by silica gel flash column chromatography using a ternary gradient from hexanes/ CH_2Cl_2 (80/20) to CH₂Cl₂/MeOH (95/5). δH (400 MHz; CDCl₃) 8.97 (1H, s, 6-N=CH) 8.52 (1H, s, H-2), 8.12 (1H, s, H-8), 7.47 (2H, br d, 2 x H_{meta}, phenyl, DMTr), 7.36 (4H, dd, ³J = 6.84 Hz and ⁴J = 1.92 Hz, 4 x H_{ortho}, methoxyphenyl, DMTr), 7.31 - 7.22 (3H, m, partly covered by solvent, 3 x H, phenyl, DMTr), 6.78 $(4H, dd, {}^{3}J = 8.00 Hz and {}^{4}J = 0.84 Hz, 4 x H_{meta}$, methoxyphenyl, DMTr), 6.20 (1H, d, {}^{3}J = 4.32 Hz, H-1'), 4.47 (1H, dd, ${}^{3}J$ = 5.00 Hz and ${}^{3}J$ = 4.54 Hz, H-2'), 4.31 (1H, dd, ${}^{3}J$ = 5.04 Hz and ${}^{3}J$ = 5.08 Hz, H-3'), 3.99 (1H, dd, ³J = 4.48 Hz and ³J = 3.56 Hz, H-4'), 3.87-3.73 (2H, m, 2 x H, 2'-O-CH₂), 3.81 and 3.80 (2 x 3H, 2 x s, 2 x O-CH₃, DMTr), 3.48 (2H, AB-system with A dd and B dd, ${}^{3}J = 3.24$ Hz and ${}^{2}J = 10.56$ Hz, 2 x H-5'), 3.28 and 3.23 (2 x 3H, 2 x s, 2 x N-CH₃), 2.28 (2H, dt, ³J = 7.20 Hz and ⁴J = 2.72 Hz, 2'-O-CH₂-CH₂-CH₂), 1.95 (1H, t, ⁴J = 2.64 Hz, C=CH), 1.81 (2H, br m, 2'-O-CH₂-CH₂). δC (100 MHz; CDCl₃) 161.6 (C-6), 158.5 (2 x C_{para}, methoxyphenyl, DMTr), 156.1 (C=N, formimide), 152.9 (C-2 and C-4), 144.6 (C_{ipso}, phenyl, DMTr), 140.1 (C-8), 135.7 (2 x C_{ipso}, methoxyphenyl, DMTr), 130.0 (2 x C_{meta}, phenyl, DMTr), 128.2 (2 x Cortho, phenyl, DMTr), 127.9 (4 x Cortho, methoxyphenyl, DMTr), 126.9 (Cpara, phenyl, DMTr), 126.2 (C-5), 113.2 (4 x C_{meta}, methoxyphenyl, DMTr), 86.7 (C-1'), 83.9 (C-2'), 82.2 (C-4'), 81.8 (C=CH), 70.2 (C-3'), 69.5 (C=CH), 69.3 (2'-O-CH₂), 63.3 (C-5'), 55.2 (2 x O-CH₃), 41.3 (N-CH₃), 35.2 (N-CH₃), 28.0 (2'-O-CH₂-CH₂), 15.1 (2'-O-CH₂-CH₂-CH₂). ESI-MS: positive mode 691.03 ([M+H]⁺). Calc.: 690.62.

(2*R*,3*R*,4*R*,5*R*)-2-((bis(4-Methoxyphenyl)(phenyl)methoxy)methyl)-5-(6-(-((dimethylamino)methylene)amino)-9*H*-purin-9-yl)-4-(pent-4-yn-1-yloxy)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (2)



In a dry vessel, compound **10** (1.32 g, 2.0 mmol), dried overnight *in vacuo*, was dissolved in a mixture of dry CH_2Cl_2 (5 mL) and dry DIPEA (0.39 g, 3.0 mmol) at 0 °C. To the solution was added 2-cyanoethyl diisopropyl-phosphoramidochloridite (0.71 mL, 3.0 mmol). After 2 min the ice bath was

removed and the mixture was stirred at rt for 3 h. The solution was diluted with CH_2Cl_2 (30 mL) and extracted once with saturated ice-cold aq. NaCl solution (10 mL) and twice with ice-cold H_2O (each 10 mL). The organic layer was dried with Na_2SO_4 and concentrated by rotary evaporation to ca. 5 mL. The residue was purified by flash silica gel chromatography using a gradient of n-hexane/CH₂Cl₂ (90:10) to CH_2Cl_2 . The solvents contained 0.5 % TEA. Yield: 1.48 g (83 %). δP (162 MHz; CDCl₃) 150.67 and 149.94. ESI-MS: positive mode 891.32 ([M+H]⁺). Calc.: 890.95.

Synthesis and characterization of *N*-(3-bromopropyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8naphthyridine-3-carboxamide (j)⁶



Scheme S3: Synthesis of nalidix acid amide. Reagents and conditions: a) isobutylchloroformate, N-methyl morpholine, dry CH₂Cl₂, -25 °C to rt, 3 h.

N-(3-Bromopropyl)-1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxamide (j)

Nalidixic acid (1.16 g, 5 mmol) and N-methylmorpholine (0.5 g, 5 mmol) were dissolved in 20 mL of dry CH_2Cl_2 . The solution was cooled to – 25 °C and activation of the carboxylic acid was effected by addition of 0.65 mL (0.68 g, 5 mmol) of isobutylchloroformate. After 1 min, a solution of 3-bromopropyl amine hydrobromide (2.19 g, 10 mmol), pre-dissolved in 20 mL of a mixture of N-methylmorpholine and CH₂Cl₂ (1:10) on ice, was added, and the reaction was allowed to warm slowly to ambient temperature and stirred vigorously for 3 h. The volatiles were removed by rotary evaporation, the product was dissolved in 50 mL of chloroform, washed three times with 10 % aq. NaHSO₄ solution (20 mL each), three times with aq. NaHCO₃ solution (20 mL each) and three times with water (20 mL each). The organic layer was dried with Na₂SO₄ and evaporated. The product (1.29 g, 73 %). was dissolved in CH₂Cl₂ and purified by column chromatography using a gradient of CH₂Cl₂ to CH₂Cl₂/MeOH (90:10). δH (400 MHz; CDCl₃) 10.04 (1H, s, H-2), 8.92 (1H, s, CONH), 8.66 (1H, d, ³J = 8.12 Hz, H-5), 7.32 (1H, d, ³J = 8.16 Hz, H-6), 4.58 (2H, q, ³J = 7.16 Hz, N1-<u>CH₂</u>), 3.66 (2H, t, ³J = 4.40 Hz, CONH-<u>CH₂</u>), 3.52 (2H, t, ³J = 6.68 Hz, CH₂-Br), 2.72 (3H, s, 7-CH₃), 2.22 (2H, tt, ³J = 6.60 Hz, and ³J = 4.48 Hz, CONH-CH₂-<u>CH₂</u>), 1.52 (3H, t, ³J = 7.16 Hz, N1-CH₂-CH₃). δC (100 MHz, CDCl₃) 177.0 (C-4), 167.8 (C-8a), 155.0 (CONH), 148.1 (C-2), 147.3 (C-7), 136.5 (C-5), 134.9 (C-6), 121.4 (C-4a), 111.6 (C-3), 46.8 (N1-CH₂), 37.6 (CONH-CH₂), 32.8 (CONH-CH₂-CH₂), 30.9 (CH₂-Br), 25.2 (7-CH₃), 15.3 (N1-CH₂-<u>CH₃</u>). ESI-MS: positive mode 351.50 and 353.56 ([M+H]⁺). Calc.: 352.23.

Synthesis, purification and characterization of oligonucleotides

Oligonucleotides were synthesized with standard phosphoramidites on a 50 nmol scale. In case of modified phosphoramidite **2** the coupling time was prolonged to 3 x 4 min. After synthesis or post-synthetic modification by CuAAC (see below), the oligonucleotides were deprotected with gas. methylamine at 60-70 °C for 2 h. Oligonucleotides modified with **d** were deprotected in 500 μ L of 40 % aq. methylamine and 30 % aqueous ammonia (1:1 vol/vol) for 4 h at rt. The solid support was filtered off and washed with 3 x 100 μ L H₂O/EtOH (1:1) and 50 μ L of 1N Tris-base. All solutions were combined and evaporated to dryness in a SpeedVac. The residue was dissolved in 200 μ L of water and purified DMT-on by RP-HPLC. The isolated product fractions were dried in a SpeedVac, treated for 30 min with a 40 % aqueous solution of acetic acid at rt, dried in a SpeedVac and purified DMT-off by RP-HPLC.

Copper catalyzed alkyne-azide cycloaddition (CuAAC)⁷

The CuAAC reaction was performed as previously described, with a modification of the protocol that allowed us to employ benzylic and aliphatic halides as starting material.⁷

a) Preparation of azides from halides for CuAAC: 10 μ mol of a halide **b**-**j** was dissolved in 800 μ L of DMF in an Eppendorf tube. To this solution were added 100 μ L of an aqueous Na-azide solution (12 μ mol, 0.8 mg/100 μ L) and 100 μ L of TBAI in DMF (2 μ mol, 0.75 mg/100 μ L). Both Na-azide and TBAI solutions were prepared as stock solutions immediately before the substitution reaction. The reaction was shaken for 1 h at rt except for halides **e** (theophylline) and **j** (nalidixic acid amide) which were shaken at 70 °C.

b) Parallel alkyne-azide cycloaddition: the CPG carrying ca. 50 nmol of an alkynyl-modified 2'methoxyRNA **3a** - **6a** was suspended in 1000 μ L of H₂O/MeOH (1:1). The suspension was pipetted up and down several times and each 100 μ L of the homogenized suspension was carefully distributed to 9 wells of a 96-well deep well plate. Subsequently, 380 μ L of H₂O/MeOH (1:1), an azide **b**-j (1 μ mol in 100 μ L of DMF/H₂O 9:1, see above), TBTA (0.1 μ mol, 0.53 mg in 20 μ L of DMF), Na-ascorbate (0.1 μ mol, 0.2 mg in 10 μ L of H₂O) and CuSO₄ (0.01 μ mol, 0.0125 mg in 10 μ L of H₂O) were added to the suspension in this order. All solutions had been prepared as stock solutions immediately before the CuAAC reaction was started. The plate was sealed and the reaction mixture was shaken at 45 °C overnight. Then, the CPGs were filtered off using a 96-well filter plate and washed subsequently with each 3 x 0.2 mL of DMF, 0.1 N aqueous EDTA, DMF, MeCN and CH₂Cl₂. The CPGs containing oligonucleotides modified with d were filtered off separately using filter columns. The filter plate and filter columns were dried shortly *in vacuo*, and the oligonucleotides were submitted to work-up procedures as described above.

sequence:	3a - 6a	3a	4a	5a	6a	
	calc.	found	found	found	found	
	5301.76	5300.97	5300.86	5301.02	5301.08	
sequence:	3b-j - 6b-j	3b-j	4b-j	5b-j	6b-j	
	calc.	found	found	found	found	
b	5527.01	5526.11	5526.31	5526.27	5525.54	
C	5468.89	5468.07	5468.10	5487.00	5487.10	
d	5536.06	5535.79	5535.16	5535.25	5535.15	
е	5550.99	5550.18	5550.03	5549.91	5550.05	
f	5485.96	5485.20	5484.45	5485.23	5485.19	
g	5501.96	5501.13	5501.73	5501.29	5500.32	
h	5487.97	5487.25	5486.02	5487.24	5487.10	
i	5536.02	5535.19	5535.14	5535.30	5535.22	
j	5616.10	5615.30	5615.30	5615.28	5615.31	

Table S2: Calculated and found masses of antimiR-122-small molecule conjugates 3a-j – 6a-j.

Table S3: Extinction coefficients of small molecules b-j at 260 nm

(http://webbook.nist.gov/chemistry	ıl)
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No.	b	с	d	е	f	g	h	i	j ⁶
ε [l/mol x cm]	3100	10000	1000	6000	1600	6300	3300	2500	23000

HPLC-traces and MS deconvolution from antimiR-small molecule conjugates:



















compound 3e







compound 3g



compound 3h



compound 3i















min











compound **4d**













compound **4j**



compound **5a**







compound 5c



compound 5d



compound 5e















compound 5i







compound 6a







compound **6c**



compound **6d**











compound 6g







compound 6i



compound 6j

Synthesis and characterization of compounds 7 and 8

Compounds **7** and **8** were synthesized according to a previously described procedure making use of the in situ generated azides of fragments **h** and **i** (Scheme 1), which were positioned as indicated in the sequences (Table 1).⁴



Literature:

- 1. A. V. Tataurov, Y. You, R. Owczarzy, Biophys. Chem., 2008, 133, 66.
- 2. J. Imig, A. Brunschweiger, A. Brummer, B. Guennewig, N. Mittal, S. Kishore, P. Tsikrika, A. P. Gerber, M. Zavolan, J. Hall, *Nat. Chem. Biol.*, 2015, **11**, 107.
- J. A. Boos, D. W. Kirk, M. L. Piccolotto, W. Zuercher, S. Gfeller, P. Neuner, A. Dattler, W. L. Wishart, F. Von Arx, M. Beverly, J. Christensen, K. Litherland, E. van de Kerkhof, P. J. Swart, T. Faller, A. Beyerbach, D. Morrissey, J. Hunziker, I. Beuvink, *Nucleic Acids Res.*, 2013, 41, e145.
- 4. U. Pradere, A. Brunschweiger, L. F. Gebert, M. Lucic, M. Roos, J. Hall, *Angew. Chem. Int. Ed. Engl.*, 2013, **52**, 12028.
- 5. A. M. Jawalekar, N. Meeuwenoord, J. S. Cremers, H. S. Overkleeft, G. A. van der Marel, F. P. Rutjes, F. L. van Delft, *J. Org. Chem.*, 2008, **73**, 287.
- 6. R. Singh, Geetanjali, Synthesis, 2005, 14, 2315.
- 7. T. Yamada, C. G. Peng, S. Matsuda, H. Addepalli, K. N. Jayaprakash, M. R. Alam, K. Mills, M. A. Maier, K. Charisse, M. Sekine, M., et al. *J. Org. Chem.*, 2011, **76**, 1198-1211.