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Supplemental information

Investigating discovery strategies and pharmacological properties of stereodefined phosphorodithioate LNA gapmers

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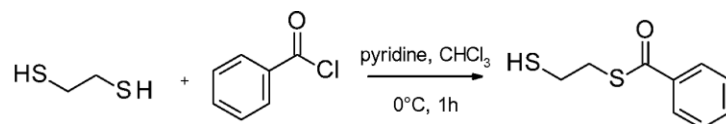
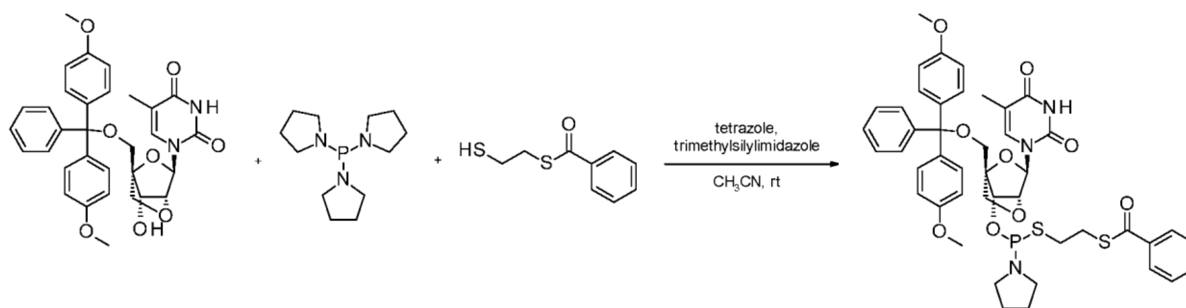
LNA thiophosphoramidite monomer synthesis:

S-(2-sulfanylethyl) benzenecarbothioate



To a solution of 1,2-ethanedithiol (133.6 mL, 1592 mmol, 1 eq) and pyridine (64.4 mL, 796 mmol, 0.5 eq) in chloroform (200 mL) was added benzoyl chloride (92.4 mL, 796 mmol, 0.5 eq) in chloroform (200 mL) dropwise for 1 hr, and the reaction was stirred at 0 °C for 1 hr. The mixture was washed with water (300 mL) and brine (300 mL). The organic phase was dried over Na_2SO_4 and concentrated to a yellow oil. The oil was distilled (135~145 °C) to afford S-(2-sulfanylethyl) benzenecarbothioate (40 g, 202 mmol, 13% yield) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, J = 7.34 Hz, 2H), 7.53-7.64 (m, 1H), 7.47 (t, J = 7.58 Hz, 2H), 3.31 (t, J = 7.34 Hz, 2H), 2.77-2.86 (m, 2H), 1.70 (t, J = 8.56 Hz, 1H).

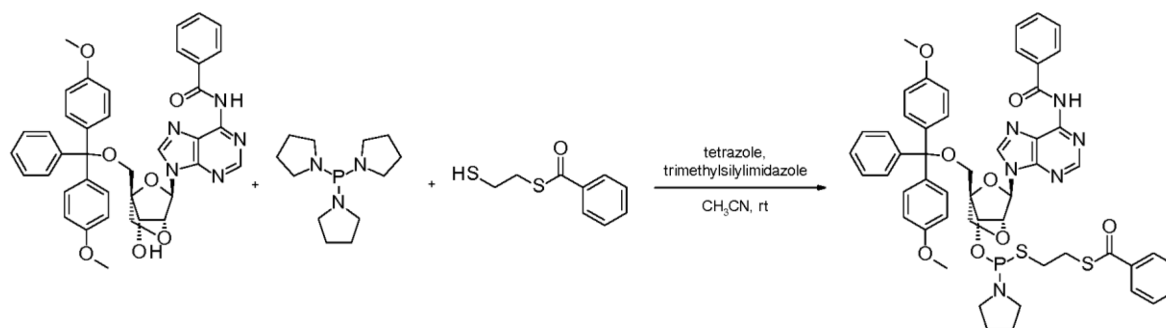
S-[2-[[(1R,3R,4R,7S)-l-[[bis(4-methoxyphenyl)-phenyl-methoxy] methyl]-3-(5-[2-methyl-2,4-dioxo-pyrimidin-l-yl)-2,5-dioxabicyclo[2.2.1]heptan-7-yl]oxy-pyrrolidin-1-yl-phosphanyl] sulfanylethyl] benzenecarbothioate



1-[(1R,4R,6R,7S)-4-[[bis(4-methoxyphenyl)-phenyl-methoxy]methyl]-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptan-6-yl]-5-methyl-pyrimidine-2,4-dione (2.29 g, 4.00 mmol, 1.0 eq) was dissolved in 60 mL of anhydrous dichloromethane to which a spatula of 3 Å molecular sieves was added. Tripyrrolidin-1-ylphosphane (960 mg, 3.98 mmol, 0.99 eq) was added via syringe followed by seven 0.1 mmol aliquots of tetrazole (7 x 0.4 mL of a 0.5 M solution in

anhydrous acetonitrile stored over 3 Å molecular sieves) at 2 min intervals. N-trimethylsilylimidazole (56.0 mg, 0.400 mmol, 0.1 eq) was then added to the reaction. After 5 min, tetrazole (21.6 mL of a 0.5 M solution in anhydrous acetonitrile) was added, immediately followed by the addition of S-(2-sulfanylethyl) benzenecarbothioate (1.04 g, 5.24 mmol, 1.31 eq). The reaction was allowed to proceed for 120 sec. Four identical batches of the reaction were combined and quenched by pouring the solution into 600 mL of dichloromethane containing 40 mL of triethylamine. The mixture was immediately washed with saturated sodium bicarbonate (800 mL) followed by 10% sodium carbonate (2 x 800 mL) and brine (800 mL). The organic layer was dried over Na_2SO_4. After 10-15 min the drying agent was removed by filtration. Triethylamine (40 mL) was added to the solution which was concentrated using a rotary evaporator to a syrup. The syrup was dissolved in toluene (200 mL) and triethylamine (40 mL), and this solution was pipetted into 4500 mL of vigorously stirred heptane to precipitate the fluffy white product. After most of the heptane was decanted, the white precipitate was collected by filtration through a medium sintered glass funnel and subsequently dried under vacuum to give a white solid. The solid was purified by prep-HPLC (Phenomenex Gemini C18, 250x50 mm, 10 mm column, 0.05% ammonium hydroxide in water / CH_3CN), and freeze-dried to afford 4.58 g of target compound as a white solid. ^{31}P NMR (162 MHz, CD_3CN) δ 167.6, 164.2. ^{1}H NMR (400 MHz, CD_3CN) δ 9.16 (br s, 1H), 7.93 (t, *J* = 7.41 Hz, 2H), 7.60-7.71 (m, 1H), 7.45-7.57 (m, 4H), 7.24-7.45 (m, 7H), 6.90 (d, *J* = 8.93 Hz, 4H), 5.53-5.63 (m, 1H), 4.41-4.64 (m, 2H), 3.74-3.88 (m, 8H), 3.39-3.63 (m, 2H), 3.03-3.32 (m, 5H), 2.77-2.94 (m, 2H), 1.66-1.84 (m, 4H), 1.54-1.66 (m, 3H).

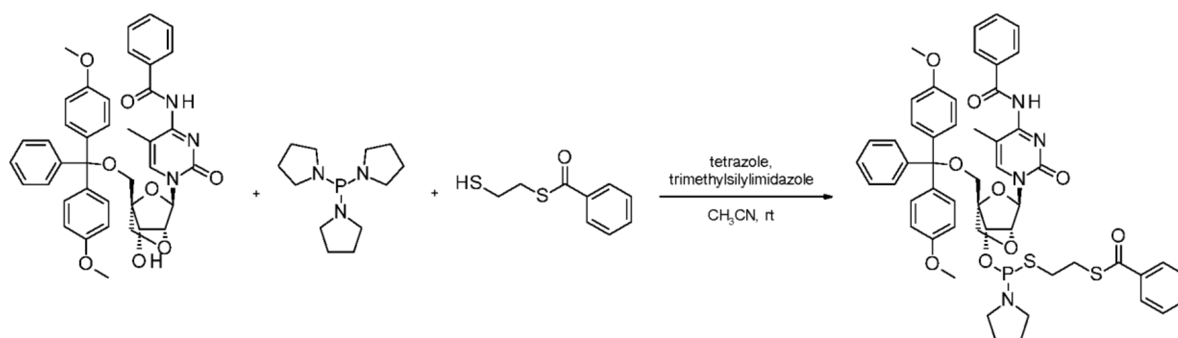
S-[2-[[(1R,3R,4R,7 S)-3-(6-benzamidopurin-9-yl)- 1- [[bis(4-methoxyphenyl)-phenyl-methoxy] methyl]-2,5-dioxabicyclo-[2.2.1]-heptan-7-yl]oxy-pyrrolidin-1-ylphosphanyljsulfanylethyl]benzenecarbothioate



N-[9-[(1R,4R,6R,7S)-4-[[bis(4-methoxyphenyl)-phenyl-methoxy]methyl]-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptan-6-yl]purin-6-yl]benzamide (2.74 g, 4.00 mmol, 1.0 eq) was dissolved in 60 mL of anhydrous dichloromethane to which a spatula of 3 Å molecular sieves was added. Tripyrrolidin-1-ylphosphane (960 mg, 3.98 mmol, 0.99 eq) was added via syringe followed by seven 0.1 mmol aliquots of tetrazole (7 * 0.4 mL of a 0.5 M solution in anhydrous acetonitrile stored over 3 Å molecular sieves) at 2 min intervals. 1-(trimethylsilyl)-1H-imidazole (56.0 mg, 0.400 mmol, 0.1 eq) was then added to the reaction. After 5 min, tetrazole (21.6 mL of a 0.5 M solution in anhydrous acetonitrile) was added, immediately followed by the addition of S-(2-sulfanylethyl) benzenecarbothioate (1.04 g, 5.24 mmol, 1.31 eq). The reaction was allowed to proceed for 120 s. Four identical batches of the reaction were united and quenched by pouring the solution into 600 mL of dichloromethane containing 40 mL of triethylamine. The mixture was immediately washed with saturated sodium bicarbonate (800 mL) followed by 10% sodium carbonate (2 x 800 mL) and brine (800 mL). The organic layer was dried over Na_2SO_4. After 10-15 mins the drying agent was removed by filtration. Triethylamine (10 mL) was added to the solution which was concentrated using a rotary evaporator to a syrup. The syrup was dissolved in toluene (100 mL) and triethylamine (20 mL), and this solution was pipetted into 4500 mL of vigorously stirred heptane to precipitate the fluffy white product. After most of the heptane was decanted, the white precipitate was collected by filtration through a medium sintered glass funnel and subsequently dried under vacuum to give a white solid. The solid was purified by prep-HPLC (Phenomenex Gemini C18,

250x50 mm, 10 mm column, 0.05% ammonium hydroxide in water / CH_3CN), and freeze-dried to afford 5.26 g of target compound as a white solid. ^{31}P NMR (162 MHz, CD_3CN) d 165.6, 164.7. ^{1}H NMR (400 MHz, CD_3CN) δ 8.56 (d, J = 10.76 Hz, 1H), 8.24 (d, J = 10.27 Hz, 1H), 7.82-7.93 (m, 2H), 7.71-7.80 (m, 2H), 6.92-7.54 (m, 14H), 6.68-6.83 (m, 4H), 6.03 (d, J = 6.48 Hz, 1H), 4.70-4.90 (m, 2H), 3.81-3.98 (m, 2H), 3.59-3.68 (m, 7H), 3.25-3.47 (m, 2H), 2.81-3.02 (m, 6H), 2.56-2.81 (m, 2H), 1.44-1.72 (m, 4H).

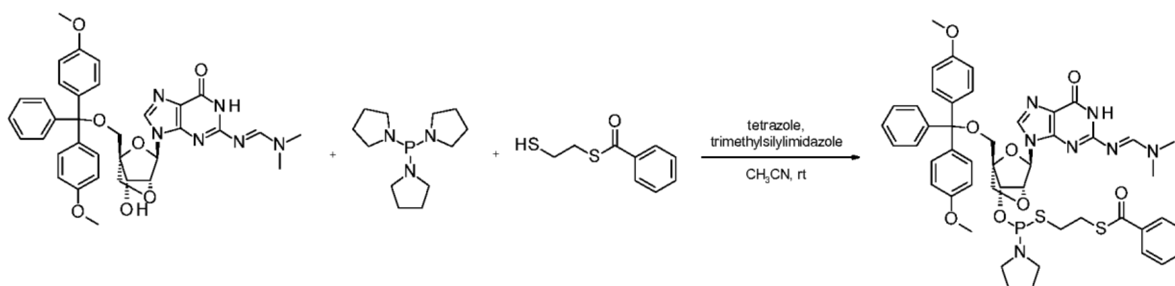
S-[2-[[(1R,3R,4R,7 S)-3-(4-benzamido-5-methyl-2-oxo-pyrimidin-1-yl)-1-[[bis(4 methoxyphenyl)-phenyl-methoxy] methyl] -2,5-dioxabicyclo [2.2.1] heptan-7-yl] oxypyrrolidin-1-yl-phosphanyl] sulfanylethyl] benzenecarbothioate



N-[1-[(1R,4R,6R,7S)-4-[[bis(4-methoxyphenyl)-phenyl-methoxy]methyl]-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptan-6-yl]-5-methyl-2-oxo-pyrimidin-4-yl]benzamide (2.70 g, 4.00 mmol, 1.0 eq) was dissolved in 60 mL of anhydrous dichloromethane to which a spatula of 3 Å molecular sieves was added. Tripyrrolidin-1-ylphosphane (965 mg, 4.00 mmol, 1.0 eq) was added via syringe followed by seven 0.1 mmol aliquots of tetrazole (7 x 0.4 mL of a 0.5 M solution in anhydrous acetonitrile stored over 3 Å molecular sieves) at 2 min intervals. 1-(trimethylsilyl)-1H-imidazole (56.0 mg, 0.400 mmol, 0.1 eq) was then added to the reaction. After 5 min, tetrazole (21.6 mL of a 0.5 M solution in anhydrous acetonitrile) was added, immediately followed by the addition of S-(2-sulfanylethyl) benzenecarbothioate (1.04 g, 5.24 mmol, 1.31 eq). The reaction was allowed to proceed for 120 sec. Four identical batches of the reaction were quenched and united by pouring the solution into 600 mL of dichloromethane

containing 40 mL of triethylamine. The mixture was immediately washed with saturated sodium bicarbonate (800 mL) followed by 10% sodium carbonate (2 x 800 mL) and brine (800 mL). The organic layer was dried over Na_2SO_4. After 10-15 min the drying agent was removed by filtration. Triethylamine (40 mL) was added to the solution which was concentrated using a rotary evaporator to a syrup. The syrup was dissolved in toluene (100 mL) and triethylamine (30 mL), and this solution was pipetted into 4500 mL of vigorously stirred heptane to precipitate the fluffy white product. After most of the heptane was decanted, the white precipitate was collected by filtration through a medium sintered glass funnel and subsequently dried under vacuum to give a white solid. The solid was purified by prep-HPLC (Phenomenex Gemini C18, 250x50mm, 10 mm column, 0.05% ammonium hydroxide in water / CH_3CN) and freeze-dried to afford 2.05 g of target compound as a white solid. ^{31}P NMR (162 MHz, CD_3CN) δ 171.2, 167.4. ^{1}H NMR (400 MHz, CD_3CN) δ 8.18-8.32 (m, 2H), 7.81-7.93 (m, 3H), 7.35-7.60 (m, 14H), 7.17-7.35 (m, 2H), 6.93 (d, J = 8.93 Hz, 4H), 5.65 (d, J = 15.04 Hz, 1H), 4.56-4.72 (m, 2H), 3.69-3.90 (m, 8H), 3.45-3.61 (m, 2H), 3.03-3.26 (m, 6H), 2.76-3.02 (m, 2H), 1.65-1.93 (m, 7H).

S-[2-[[(lR,3R,4R,7S)-l-[[bis(4-methoxyphenyl)-phenyl-methoxy]methyl]-3-[2-[(E)-dimethylaminomethyleneamino]-6-oxo-lH-purin-9-yl]-2,5-dioxabicyclo[2.2.1]heptan-7-yl]oxy-pyrrolidin-l-yl-phosphanyl]sulfanylethyl] benzenecarbothioate



N'-[9-[(1R,4R,6R,7S)-4-[[bis(4-methoxyphenyl)-phenyl-methoxy]methyl]-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptan-6-yl]-6-oxo-1H-purin-2-yl]-N,N-dimethyl-formamidine (2.62 mg, 4.00 mmol, 1.0 eq) was dissolved in 200 mL of anhydrous dichloromethane to which a spatula of 3 Å molecular sieves was added. Tripyrrolidin-1-ylphosphane (965 mg, 4.00 mmol, 1.0 eq) was added via syringe followed by seven 0.1 mmol aliquots of tetrazole (7 x 0.4 mL of a 0.5 M solution in anhydrous acetonitrile stored over 3 Å molecular sieves) at 2 min intervals. 1-(trimethylsilyl)-1H-imidazole (56.0 mg, 0.400 mmol, 0.1 eq) was then added to the reaction. After 5 min, tetrazole (21.6 mL of a 0.5 M solution in anhydrous acetonitrile) was added, immediately followed by the addition of S-(2-sulfanylethyl) benzenecarbothioate (1.04 g, 5.24 mmol, 1.31 eq). The reaction was allowed to proceed for 180 s.

Four identical batches were combined and quenched by pouring the solutions into 600 mL of dichloromethane containing 40 mL of triethylamine. The mixture was immediately washed with saturated sodium bicarbonate (800 mL) followed by 10% sodium carbonate (2 * 800 mL) and brine (800 mL). The organic layer was dried over Na_2SO_4. After 10-15 min the drying agent was removed by filtration. Triethylamine (40 mL) was added to the solution which was concentrated using a rotary evaporator to a syrup. The syrup was dissolved in toluene (100 mL) and triethylamine (30 mL), and this solution was pipetted into 4500 mL of vigorously stirred heptane to precipitate the fluffy white product. After most of the heptane was decanted, the white precipitate was collected by filtration through a medium sintered glass funnel and subsequently dried under vacuum to give a white solid. The solid was purified by prep-HPLC (Phenomenex Gemini C18, 250x50mm, 10 mm column, 0.05% ammonium hydroxide in water / CH_3CN) and freeze-dried to afford 3.82 g of target compound as a yellow solid. ^{31}P NMR (162 MHz, CD_3CN) δ 167.1, 162.2. ^{1}H NMR (400 MHz, CD_3CN) δ 9.36 (br s, 1H), 8.63 (d, *J* = 16.51 Hz, 1H), 7.78-8.00 (m, 3H), 7.66 (t, *J* = 7.62 Hz, 1H), 7.42-7.57 (m, 4H), 7.24-7.40 (m,

7H), 6.89 (d, J = 8.68 Hz, 4H), 5.92-5.98 (m, 1H), 4.72-4.97 (m, 2H), 3.86-4.05 (m, 2H), 3.78 (2s, 6H), 3.27-3.70 (m, 3H), 2.87-3.20 (m, 12H), 2.67-2.82 (m, 2H), 1.54-1.79 (m, 4H).

SUPPLEMENTARY FIGURES

A

ID	Design[a]	IC_{50} (μM)	Tm[b] (°C)
Reference	G•C•a•a•g•c•a•t•c•c•t•G•T	2.9	56.2
ASO-1	G•C•a•a•g•c•a•t•c•c•t•G^{o}T	3.3	56.1
ASO-2	G^{o}C•a•a•g•c•a•t•c•c•t•G•T	1.7	57.6
ASO-3	G^{o}C•a•a•g•c•a•t•c•c•t•G^{o}T	2.1	56.7
ASO-4	G^{o}C^{o}a•a•g•c•a•t•c•c•t•G^{o}T	2.3	56.3

B

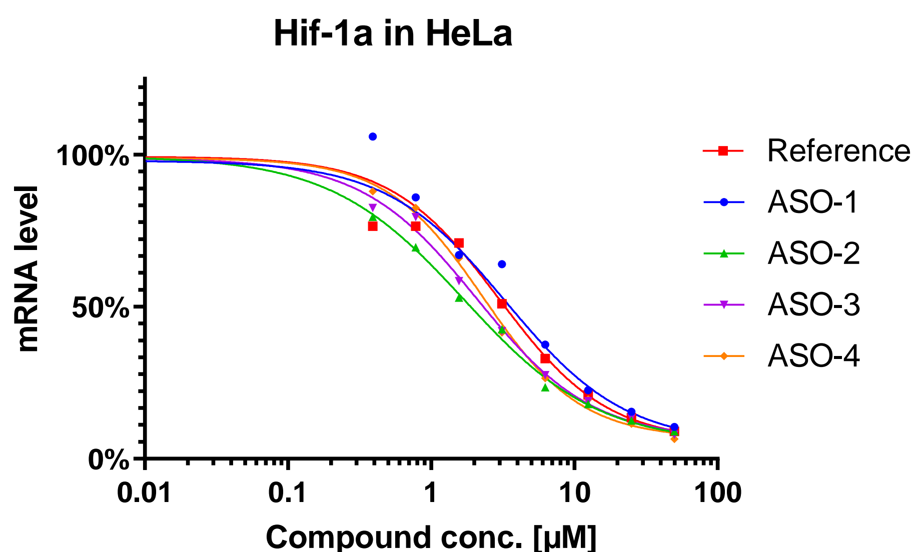


Figure S1. *In vitro Hif-1α* mRNA target reduction in HeLa cells after 72h of gapmer ASOs exposure under gymnotic uptake. **(A)** Design, antisense activity and Tm. Positions of phosphorodithioate (PS_2, ^{o}) are shown while the rest of ASO is stereorandom PS modified. [a] Upper case = LNA nucleotide; lower case = DNA nucleotide; stereorandom PS (•); phosphorodithioate (^{o}); [b] Melting temperature to complementary RNA (Tm); Complementary RNA strand for Tm measurement: ACAGGAUGCUUGC. **(B)** Dose-response curves for reducing *Hif-1α* mRNA in HeLa cells.

A

ID	Design[a]	IC_{50} (µM)	Tm[b] (°C)
Reference	G•A•G•t•t•a•c•t•t•g•c•c•a•A•C•T	0.27	62.9
ASO-1	G•A•G•t•t•a•c•t•t•g•c•c•a•A•C°T	0.27	62.9
ASO-2	G°A•G•t•t•a•c•t•t•g•c•c•a•A•C•T	0.24	62.9
ASO-3	G°A•G•t•t•a•c•t•t•g•c•c•a•A•C°T	0.33	63.0
ASO-4	G°A•G•t•t•a•c•t•t•g•c•c•a•A°C°T	0.34	61.9
ASO-5	G°A°G•t•t•a•c•t•t•g•c•c•a•A•C°T	0.36	61.5
ASO-6	G°A°G•t•t•a•c•t•t•g•c•c•a•A°C°T	0.42	61.2
ASO-7	G°A°G°t•t•a•c•t•t•g•c•c•a•A°C°T	0.46	60.5

B

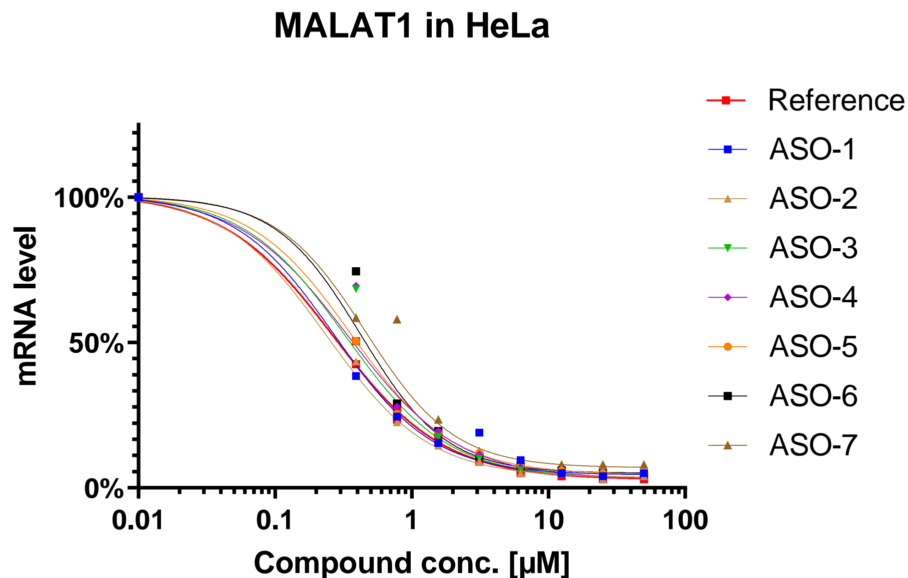
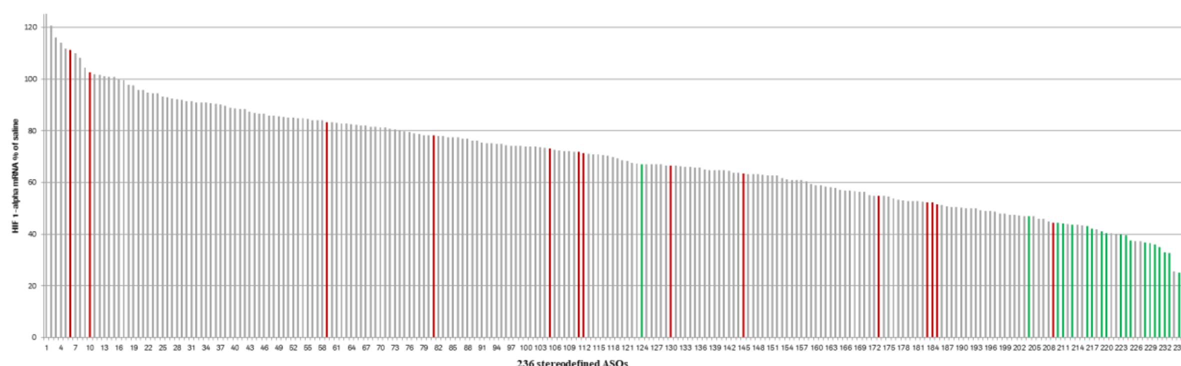


Figure S2. *In vitro Malat1* lncRNA target reduction in HeLa cells after 72h of gapmer ASOs exposure under gymnotic uptake. **(A)** Design, antisense activity and Tm. Positions of phosphorodithioate (PS_2, °) are shown while the rest of ASO is stereorandom PS modified. [a] Upper case = LNA nucleotide; lower case = DNA nucleotide; stereorandom PS (•); phosphorodithioate (°); [b] Melting temperature to complementary RNA (Tm); Complementary

RNA strand for Tm measurement: AGUUGGCAAGUAACUC. **(B)** Dose-response curves for reducing *Malat1* lncRNA in HeLa cells.

A



B

ID	Stereochemistry[a]	KD[b] (5 µM) %mRNA	ID	Stereochemistry[a]	KD[b] (5 µM) %mRNA
ASO-1	RSRR**RSSR**SRSS	23	ASO-18	SSSS**RSSR**RSSS	67
ASO-2	RSRR**RSSR**RSRS	25	ASO-19	RSRRR**RSSR**RSS	46
ASO-3	SRRS**RSSR**RRSS	33	ASO-20	RSRRR**RSSR**RSS	52
ASO-4	RSSR**RSSR**SSRR	33	ASO-21	RSRRR**RSSR**RSS	52
ASO-5	RRSR**RSSR**SSSS	35	ASO-22	RSRRR**RSSR**RSS	55
ASO-6	SSRS**RSSR**SRRS	36	ASO-23	RSRRR**RSSR**RSS	64
ASO-7	SRRR**RSSR**SRRR	36	ASO-24	RSRRR**RSSR**RSS	67
ASO-8	SRSS**RSSR**SRSS	37	ASO-25	RSRRR**RSSR**RSS	71
ASO-9	RRRS**RSSR**SRRS	38	ASO-26	RSRRR**RSSR**RSS	71
ASO-10	SSRS**RSSR**RSRR	40	ASO-27	RSRRR**RSSR**RSS	73
ASO-11	SRRS**RSSR**RSRS	40	ASO-28	RSRRR**RSSR**RSS	78
ASO-12	RRRS**RSSR**SRSR	42	ASO-29	RRRSS**RSSR**SSS	83
ASO-13	SSRS**RSSR**RSSS	43	ASO-30	SRRRR**RSSR**SSS	103
ASO-14	RRSS**RSSR**RRRS	44	ASO-31	RRRRS**RSSR**SSR	111
ASO-15	SSSS**RSSR**SSSR	44			
ASO-16	SRRS**RSSR**RSSS	45			
ASO-17	SRRS**RSSR**RSSR	47			

C

ID	Stereochemistry[a]	KD[b] (5 μM) %mRNA	ID	Stereochemistry[a]	KD[b] (5 μM) %mRNA
ASO-32	SSSSRRSSSSSR	129	ASO-57	SSSRSRSSSRRR	44
ASO-33	SRRSRSRSSRSS	121	ASO-58	SSRRSRSSSSRS	44
ASO-34	RSSRRRRRSSRR	116	ASO-59	SRRSRSRRSRSS	44
ASO-35	RRSSRSRSRSRS	114	ASO-60	SRSRRSSSRRRS	43
ASO-36	SRRRSSRRSRRR	112	ASO-61	RSSSRRSRSRSS	42
ASO-37	RRRRSRSSRSSR	111	ASO-62	RRSSRSRRRRSR	40
ASO-38	SSSRSSSRRRSR	110	ASO-63	RSRSRSRRRRSS	40
ASO-39	RRRSRSRSRSSR	108	ASO-64	RRSSRRSRSRRS	37
ASO-40	RRRSSSRRSSSS	104	ASO-65	RSRRRRRRRRSS	37
ASO-41	SRRRRRSSRSSS	103	ASO-66	RSSRRSRRSRSS	26
ASO-42	RSSSSSSSSSRS	102			
ASO-43	SSSRRRRSRRRR	102			
ASO-44	RRRRRRSRSSRR	101			
ASO-45	RRSRRSRSSRRR	101			
ASO-46	SRSRSRRSRSSS	101			
ASO-47	SRRRRRRSSRSR	100			
ASO-48	SSRSSSRSRSRS	100			
ASO-49	SRSSRRRSRRSR	98			
ASO-50	SSRRRRSSSSSR	98			
ASO-51	RRSSRRRSRSSS	96			
ASO-52	SSRRSRRSSRRS	96			
ASO-53	RRRSRRRRSSRS	95			
ASO-54	RSRRRSRSRRRS	94			
ASO-55	SSRSRSRRSSRR	94			
ASO-56	SSRRRRRRSRRR	93			

Figure S3. *In vitro Hif-1α* mRNA target reduction at 5 µM in HeLa cells after 72h of exposure (gymnotic uptake) with 236 ASOs differing only for their stereochemistry (conserved heterocyclic base sequence and DNA/LNA pattern). **(A)** Target knockdown of 236 fully stereodefined analogues targeting *Hif-1α* at 5 µM in HeLa cells. 236 fully stereodefined analogues were generated (5.7% of all possible LNAs isomers). PS stereochemistry in each position is randomly selected. From the data, broad potency range was observed for all 236 fully stereodefined analogues. **(B)** Stereochemistry designs and antisense activity in HeLa cells. It was observed that RSSR stereomotif in a specific position enhances chance of finding more potent ASOs. Green color bars identify ASOs where the RSSR stereochemical motif starts at position 5 in the stereochemical description (counting left to right - $R^1S^2R^3R^4$**R^5SSR**SRSS). Red color bars identify ASOs with the RSSR stereochemical motif starting at position 6 (counting left to right – $R^1S^2R^3R^4R^5$**R^6SSR**RSS). The target knock down histogram shows that ASOs with the stereochemical motif RSSR incorporated at position 5 (green label) elicit a more potent target reduction compared ASOs where the RSSR motif starts at position 6 (red label). [a] R-configuration (R); S-configuration (S); [b] Knockdown (KD, the remaining mRNA). (**C**) Selected stereodefined ASOs with poor knockdown (ASOs 32-56) and the ones with good knockdown (ASOs 57-66) don't contain RSSR motif at a specific position can greatly impact the potency. [a] R-configuration (R); S-configuration (S); [b] Knockdown (KD, the remaining mRNA).

A

ID	Design[a]	IC50 (µM)
Reference	G•C•a•a•g•c•a•t•c•c•t•G•T	3.18
ASO-1	G°C•a•a•g•c•a•t∧c∨c∨t∧G°T	3.59
ASO-2	G°C•a•a•g•c•a∧t∨c∨c∧t•G°T	2.66
ASO-3	G°C•a•a•g•c∧a∨t∨c∧c•t•G°T	2.02
ASO-4	G°C•a•a•g∧c∨a∨t∧c•c•t•G°T	1.03
ASO-5	G°C•a•a∧g∨c∨a∧t•c•c•t•G°T	4.18
ASO-6	G°C•a∧a∨g∨c∧a•t•c•c•t•G°T	4.36

B

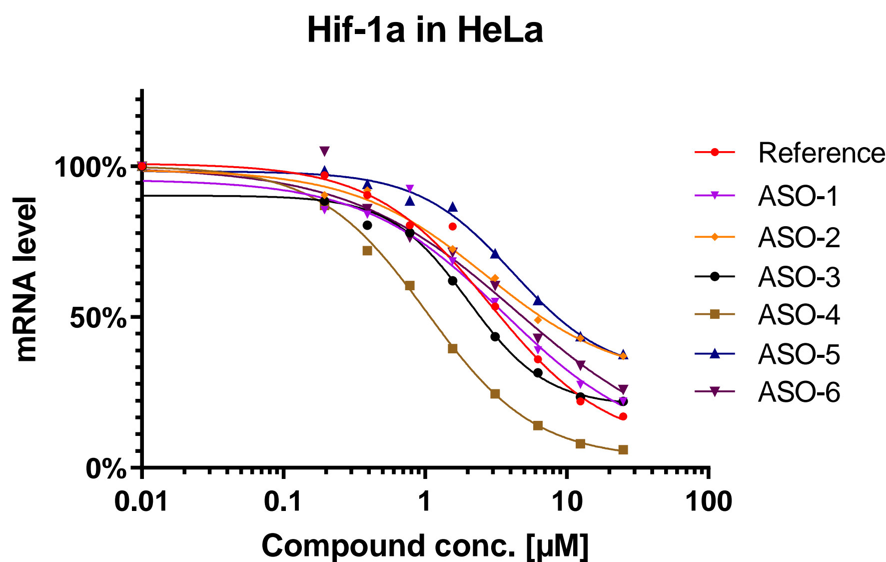


Figure S4. *In vitro Hif-1α* mRNA target reduction in HeLa after 72h of gapmer ASOs exposure under gymnotic uptake. **(A)** Design and antisense activity. Positions of phosphorodithioate (PS_2, °) and RSSR motif (∧ ∨ ∨ ∧) are shown while the rest of ASO is stereorandom PS modified. upper case = LNA nucleotide; lower case = DNA nucleotide; R-configuration (∧); S-configuration (∨); Phosphorodithioate linkage (°); Phosphorothioate linkage (•). **(B)** Dose-response curves for reducing *Hif-1α* mRNA in HeLa cells.

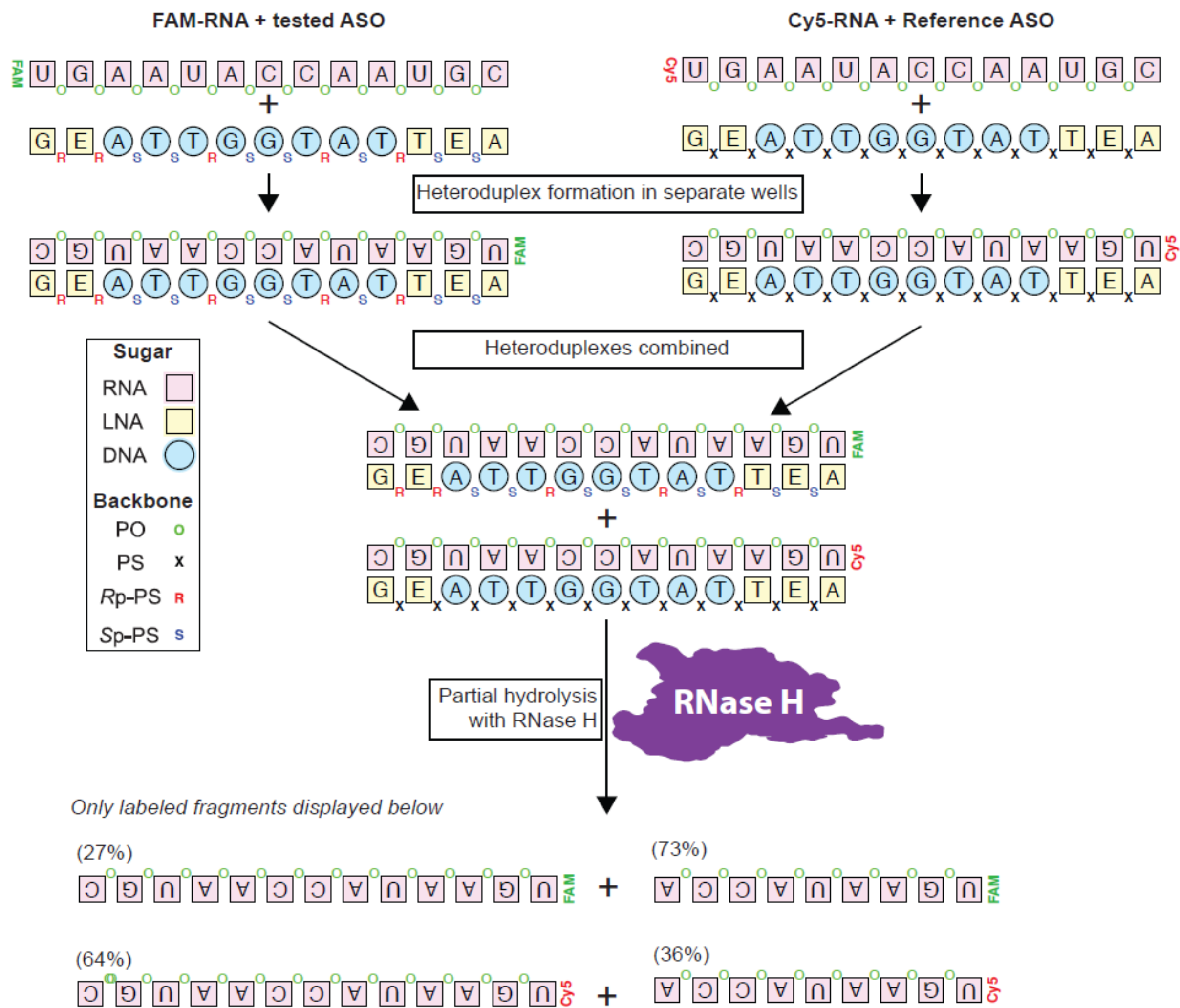


Figure S5 Demonstration of RNase H cleavage competition assay. For the heteroduplex formation, the reference ASO RTR3833 is annealed with Cy5-labeled complementary RNA strand, and the tested ASO is annealed with FAM-labeled complementary RNA strand. The equal amounts of these two heteroduplexes are mixed together, and treated with recombinant human RNase H1 solution. Reactions were terminated by addition of the stop solution. Samples were heat denatured and resolved on 15% polyacrylamide Urea-TBE gel. Gels were visualized for FAM and Cy5 channels using iBright™ FL1000 Imaging System (Thermo Fisher) and bands for full length and cleaved fractions were quantified using ImageJ.