

**Supporting Information for:**

**Design and Enhanced Gene Silencing Activity of Spherical 2'-Fluoroarabinose Nucleic Acids (FANA-SNAs)**

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**I. General:**

Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetate (EDTA), urea, 40% acrylamide/bis-acrylamide (19:1), ammonium persulfate (APS), N,N,N',N'- tetramethylethane-1,2 diamine (TEMED) and agarose were purchased from BioShop Canada Inc and used without further purification. Magnesium chloride hexahydrate and Nile Red were purchased from Sigma-Aldrich. Acetic acid, boric acid, ammonium hydroxide and 10x DPBS (with magnesium, calcium) were purchased from Fischer Scientific and used without further purification. GelRed™ nucleic acid stain was purchased from Biotium Inc. GeneRuler DNA Ladder Mix and DNA Gel Loading Dye (6X) were obtained from Thermo Scientific. 1 μmole 1000 Å universal synthesis CPG column, standard reagents used for automated DNA synthesis and Sephadex G25 (super fine DNA grade) were purchased from BioAutomation. DMT-1,12-dodecane-diol phosphoramidites were synthesized in

the lab from 1,12-dodecane-diol (Alfa Aesar) and DMTCl (AK Scientific). 1x TBE buffer is composed of 90 mM Tris, 90 mM boric acid and 2 mM EDTA with a pH ~8.3. 1x TAMg buffer is composed of 45 mM Tris, 20 mM acetic acid and 7.6 mM Mg(Cl)<sub>2</sub>, and its pH was adjusted to ~8.0 using glacial acetic acid. 1x DPBS (with magnesium and calcium) is composed of 8 mM sodium phosphate dibasic, 138 mM of sodium chloride, 1.47mM of potassium phosphate monobasic, 2.6 mM potassium chloride, 0.5 mM magnesium chloride (anhydrous) and 0.9 mM calcium chloride (anhydrous).

Cyanine 3 (Cy3) phosphoramidite and Sulfurizing reagent II were purchased from GlenResearch. Millipore water was used throughout any preparation of buffers.

Dulbecco's Modified Eagle Medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Life Technologies. Lipofectamine reagent was purchased from Invitrogen. Fetal bovine serum (FBS), 0.05% Trypsin-EDTA and sodium pyruvate were obtained from Wisent Bioproducts. The Bright-Glo Luciferase Assay system was purchased from Promega. Cell-Titer Blue assay was also purchased from Promega. The HeLa X1/5 cells stably expressing luciferase are a generous gift from Dr. Pelletier (McGill).

## II. Instrumentation:

Standard oligonucleotide synthesis was performed on solid supports using a Mermade MM6 synthesizer from Bioautomation. UV absorbance DNA quantification measurements were performed with a NanoDrop Lite spectrophotometer from Thermo Scientific. For structure assembly, Eppendorf Mastercycler 96-well thermocycler and Bio-Rad T100TM thermal cyclers were used to anneal all DNA nanoparticles. Polyacrylamide gel electrophoresis (PAGE) was performed using 20x20 cm vertical Hoefer 600 electrophoresis units, or a Bio-Rad Mini-PROTEAN® Tetra Vertical electrophoresis units. Agarose Gel Electrophoresis (AGE) were performed on Owl Mini and Owl EasyCast horizontal gel systems. Gels were imaged by BioRad ChemiDoc MP system.

Fluorescence data were measured by BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader. Multimode 8 scanning probe microscope and Nanoscope V controller (Bruker, Santa Barbara, CA) was used to acquire AFM images. Liquid Chromatography Electrospray Ionization Mass Spectrometry (LCESI-MS) was carried out using Dionex Ultimate 3000 coupled to a Bruker MaXis Impact™ QTOF. Dynamic light scattering (DLS) experiments were carried out using a DynaPro™ Instrument from Wyatt technology. Firefly Luciferase luminescence was measured using a Biotek Synergy HT plate reader. Cytotoxicity studies were performed using the CellTiter96 kit from Promega according to the manufacturer's instructions.

## III. Synthesis and purification:

Synthesis:

All solid-phase syntheses were performed on a 1 μmol scale using universal 1000 Å LCAA-CPG solid-supports. Coupling efficiencies were monitored following removal of the dimethoxytrityl (DMT) 5'-OH protecting group. DBCO-dT-CE and DMT-dodecanediol phosphoramidites were dissolved in the appropriate solvents (anhydrous acetonitrile/dichloromethane 1:3 (v/v) and acetonitrile respectively) under a nitrogen atmosphere (<0.04 ppm for oxygen, and <0.5 ppm trace moisture)

for a final concentration of 0.1M. The DMT-dodecane-diol was activated using 0.25M 5-(ethylthio)tetrazole in anhydrous acetonitrile and an extended coupling time of 5 minutes was employed. 3% dichloroacetic acid in dichloromethane was used to remove the DMT protecting group on the DNA synthesizer. Sulfurization was executed prior to capping on the DNA synthesizer using Sulfurizing Reagent II (cat.# 40-4037, Glen Research). Following synthesis, strands were cleaved from the solid-support and deprotected using 28% aqueous ammonium hydroxide solution for 20 hours at 60°C. Strands were dried under vacuum at 60°C, resuspended in Millipore H<sub>2</sub>O, then filtered with 0.22 µm centrifugal filters.

Strands were purified using polyacrylamide gel electrophoresis (PAGE) or high-performance liquid chromatography (HPLC).

#### PAGE Purification:

Aqueous solution of oligomer was mixed with an equal volume of Urea prior to loading on gel in order to assist in denaturation. oligomers were run on either 18% or 15% polyacrylamide/8M urea gels in 1xTBE for 30 minutes at 250V, followed by 60 minutes at 500V. Following gel electrophoresis, bands were imaged using a handheld UV illuminator (254nm) and excised, crushed, and suspended in ~5-10 mL H<sub>2</sub>O. This suspension was frozen by brief submersion in liquid nitrogen, before being incubated at 60°C for 16 hours. The supernatant was concentrated by evaporation, desalted using size exclusion chromatography (Sephadex G-25), and quantified (OD260) using a NanoDrop Lite spectrophotometer from Thermo Scientific.

#### HPLC Purification:

0.5 OD of sample in 20-50 µL of Millipore water were injected into a Hamilton PRP-1 5 µm 2.1x150mm column at 60°C. The mobile phases were TEAA and HPLC grade acetonitrile, with an elution gradient of 3-70% acetonitrile over 30 minutes. Strands were detected using a diode array detector monitoring absorbance at 260nm. The below table contains all the strands synthesized along with their sequences.

#### **IV. Table of Oligonucleotide sequences:**

**Bold** nucleotides are FANA modified bases. **D** represents dodecane-units. Underline is phosphorothioate linkages, while non-underlined are regular phosphate linkages.

Strand (# of bases)	Sequence (5' to 3')		Description
FANA-ASO (18)	<u>AUAUCCTTGTGTAUCCC</u>		FANA modified ASO against luciferase mRNA
FANA-SNA (18)	DDDDDDDDDD- <u>AUAUCCTTGTGTAUCCC</u>		FANA modified ASO against luciferase mRNA with 12 units of dodecane to form SNA.
FANA-(PO-dN <sub>4</sub> )-ASO (22)	(ATAT)- <u>AUAUCCTTGTGTAUCCC</u>		Same as FANA-ASO but includes a 4-nt PO spacer. Used to compare spacer vs no spacer activity.
FANA-(PO-dN <sub>4</sub> )-SNA (22)	DDDDDDDDDD-(ATAT)- <u>AUAUCCTTGTGTAUCCC</u>		Same as FANA-SNA but includes a 4-nt PO spacer. Used to compare spacer vs no spacer activity.
FANA-(PS-dN <sub>4</sub> )-ASO (22)	(ATAT)- <u>AUAUCCTTGTGTAUCCC</u>		Same as FANA-ASO but includes a 4-nt PS spacer. Used to compare to its PO counterpart.
FANA-(PS-dN <sub>4</sub> )-SNA (22)	DDDDDDDDDD-(ATAT)- <u>AUAUCCTTGTGTAUCCC</u>		Same as FANA-SNA but includes a 4-nt PS spacer. Used to compare to its PO counterpart.
FANA-(PO-dN <sub>6</sub> )-ASO (26)	(ATATTTT)- <u>AUAUCCTTGTGTAUCCC</u>		Longer spacer version of the cleavable modality.
FANA-(PO-dN <sub>6</sub> )-SNA (26)	DDDDDDDDDD-(ATATTTT)- <u>AUAUCCTTGTGTAUCCC</u>		Longer spacer version of the cleavable modality with 12 units of dodecane to form SNA.
FANA-(PS-dN <sub>6</sub> )-ASO (26)	(ATATTTT)- <u>AUAUCCTTGTGTAUCCC</u>		Longer spacer version of the non-cleavable modality.
FANA-(PS-dN <sub>6</sub> )-SNA (26)	DDDDDDDDDD-(ATATTTT)- <u>AUAUCCTTGTGTAUCCC</u>		Longer spacer version of the non-cleavable modality with 12 units of dodecane to form SNA.
FANA-Survivin-(PO-dN <sub>4</sub> )-ASO (25)	(ATAT)- <u>UGACAGAUAGGAACCU</u> GCUU		FANA modified ASO against survivin mRNA with a 4-nt PO spacer
FANA-Survivin-(PO-dN <sub>4</sub> )-SNA (25)	DDDDDDDDDD-(ATAT)- <u>UGACAGAUAGGAACCU</u> GCUU		FANA modified ASO against survivin mRNA with a 4-nt PO spacer with 12 units of dodecane to form SNA.
PS-DNA-ASO (18)	<u>ATATCCTTGTGTAATCCC</u>		Unmodified ASO against luciferase as control. All PS
PS-Survivin-ASO (21)	<u>TGACAGATAAGGAACCTGCTT</u>		Unmodified ASO against Survivin as control. All PS
PO-DNA-NegativeASO(18)	AATAATTGAATCTTCAA		Negative non targeting control with all PO backbone, used in cell assays.
PS-DNA-mmASO(18)	<u>ATATCCTAGTTGAATCCC</u>		Multiple mismatch version of PS-ASO against luciferase.
FANA-(PO-dN <sub>4</sub> )-mmASO (22)	(ATAT)- <u>AUAUCCTAGTTGAUCCC</u>		FANA modified multiple mismatch version of ASO against luciferase.
FANA-(PO-dN <sub>4</sub> )-mmSNA (22)	DDDDDDDDDD-(ATAT)- <u>ATATCCTAGTTGAATCCC</u>		FANA modified multiple mismatch version of ASO against luciferase with 12 units of dodecane to form SNA.
FANA-DNA-NegativeASO(18)	<u>AAUAATTGAATCTTCAA</u>		FANA modified negative scramble control to FANA-ASO against luciferase.
FANA-DNA-NegativeSNA(18)	DDDDDDDDDD- <u>AAUAATTGAATCTTCAA</u>		FANA modified negative scramble control to FANA-ASO against luciferase with 12 units of dodecane to form SNA.
PS-(PO-dN <sub>4</sub> )-DNA-SNA (22)	DDDDDDDDDD-(ATAT)- <u>ATATCCTTGTGTAATCCC</u>		PS modified ASO against luciferase with cleavable spacer, with 12 units of dodecane to form SNA.
FANA-APOB-(PO-dN <sub>4</sub> )-ASO (24)	(ATAT)- <u>GCCUCAGTCTGCTTCGCACC</u>		FANA modified ASO against APOB mRNA with a cleavable spacer
FANA-APOB-(PO-dN <sub>4</sub> )-SNA (24)	DDDDDDDDDD-(ATAT)- <u>GCCUCAGTCTGCTTCGCACC</u>		FANA modified SNA against APOB mRNA with a cleavable spacer

Supplementary Table 1 sequence ID table of all oligonucleotides used in this manuscript

## V. Methods

### Assembly of SNAs

To assemble the SNA, an ASO-polymer conjugate strand was annealed at the corresponding concentration (usually 10  $\mu$ M in DNA strands, 30  $\mu$ L) in Tris-Acetic acid -Magnesium\_buffer (1xTA, 7.6mM, Supplementary information) from 95 to 4  $^{\circ}$ C over 4 hours using Eppendorf Mastercycler 96-well thermocycler and Bio-Rad T100TM thermal cycler. All concentrations referred to in the manuscript are with respect to the DNA strand concentration. For example, 150nM concentration of SNA corresponds to 150nM of the strands that constitutes the SNA, which is an ASO-polymer conjugate.

### Dynamic Light Scattering (DLS)

SNAs size distribution was measured using a DynaProTM Instrument from Wyatt technology. Millipore water and 1xTAMg buffer were filtered using a 0.45  $\mu$ m nylon syringe filter before use in DLS sample preparation. A 15 $\mu$ L sample (concentration 5  $\mu$ M) was used in each measurement. Measurements were performed at 25 $^{\circ}$ C. Each measurement consisted of 20 acquisitions, with each acquisition lasting 10 seconds. Data was filtered to exclude acquisitions with a baseline above 1.01 and a SOS error above 150. A cumulants fit model was used to confirm the presence and determine the size the SNAs.

## Atomic Force Microscopy

Dry AFM was carried out using a MultiMode8™ SPM connected to a Nanoscope™ V controller (Bruker, Santa Barbara, CA). All images were obtained using ScanAsyst mode in air with AC160TS cantilevers (Nominal values: Tip radius – 9 nm, Resonant frequency – 300 kHz, Spring constant – 42 N/m) from Asylum Research. Samples were at 5 μM in TA buffer and 2.5-5μL of this solution was deposited on a freshly cleaved mica surface (ca. 7 x 7 mm) and allowed to adsorb for 1-2 seconds. Then 50 μL of 0.22 μm filtered Millipore water was dropped on the surface and instantly removed with filter paper. The surface was then washed with a further 200μL of water and the excess removed with a strong flow of nitrogen. Samples were dried under vacuum for at least 1 hour prior to imaging.

## In vitro Silencing and Viability Assays

HeLa cells were maintained in 10% FBS and antibiotic/antimycotic (AB/AM) and cultured in 5% CO<sub>2</sub> at 37 °C. Typically, cells were split in 1:4 ratio every 3 days. Luciferase knockdown assays were performed as described by Deleavey et al. (7) with minor modifications. Typically, HeLa cells were counted and seeded at a density of 10 000 cells per well in a 96-well plate. Cells were allowed to recover for 24 hours at 37 °C with 5% CO<sub>2</sub>. Then, samples were added to the appropriate well in triplets where Lipofectamine reagent was used as transfection agent and control (Invitrogen) following the vendor's procedure (using Optimem as transfection media mix). Cells were further incubated overnight (for a total of 24-72 hours post-DNA addition). All samples were added in equimolar amounts of ASO strands.

For luciferase assay, cells were washed with PBS 1× and lysed with 25 μl Glo-lysis buffer (Promega) and 25 μl of Bright-Glo luciferase reagent (Promega, USA) was added to each well. Luminescence was measured using a Biotek Synergy HT plate reader. Data was acquired with the Gen5 software suite and was analyzed and plotted using Graphpad Prism software suite.

For cytotoxicity and cell viability, the cells were incubated with a fluorescent reagent (CellTiter Blue) for 1.5 h in 5% CO<sub>2</sub> at 37 °C. Subsequently, 96-well plates were allowed to equilibrate at room temperature and the fluorescence was measured at 590 nm (Ex. 530, Em. 590) using a BioTek Synergy HT micro-plate reader. All quantifications were done using GraphPad Prism 5 software.

## Quantitative polymerase chain reaction (qPCR)

Reverse transcription and subsequent amplification were run in one pot using iTaq™ Universal SYBR Green Supermix. Each reaction used 20ng of RNA, 150nM of each primer, and had a total volume of 15μL. The relative amount of ApoB mRNA was quantified against the amount of the endogenous control mRNA of GAPDH. The primers used for ApoB and GAPDH and shown in Table 2. The thermal cycling conditions were as follows: 30 sec at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C. A negative control (distilled water), and RT-negative controls (no reverse transcriptase) were included in each run. Three independent qPCR reactions (PCR triplicates) were performed. The ΔΔCt method was used for relative quantification.

Gene	Forward	Reverse
ApoB	5'-TTTGCCCTCAACCTACCAAC-3'	5'-TGCGATCTTGTTGGCTACTG-3'
GAPDH	5'-GGAGCGAGATCCCTCCAAAAT-3'	5'-GGCTGTTGTCATACTTCTCATGG-3'

Table 2. primers used RT-qPCR experiment for the APOB in HepG2 cells.

## Flow Cytometry

HeLa cells were seeded at a density of  $5 \times 10^5$  in a 6-well plate. After 24 hours, the cells were incubated (without transfection) with the corresponding samples at a final concentration of  $1 \mu\text{M}$  for 24 hours as well. Then, cells were detached, washed and resuspended in  $1\times$  PBS and processed using FACS FORTESSA. All measurements were performed in doublets.

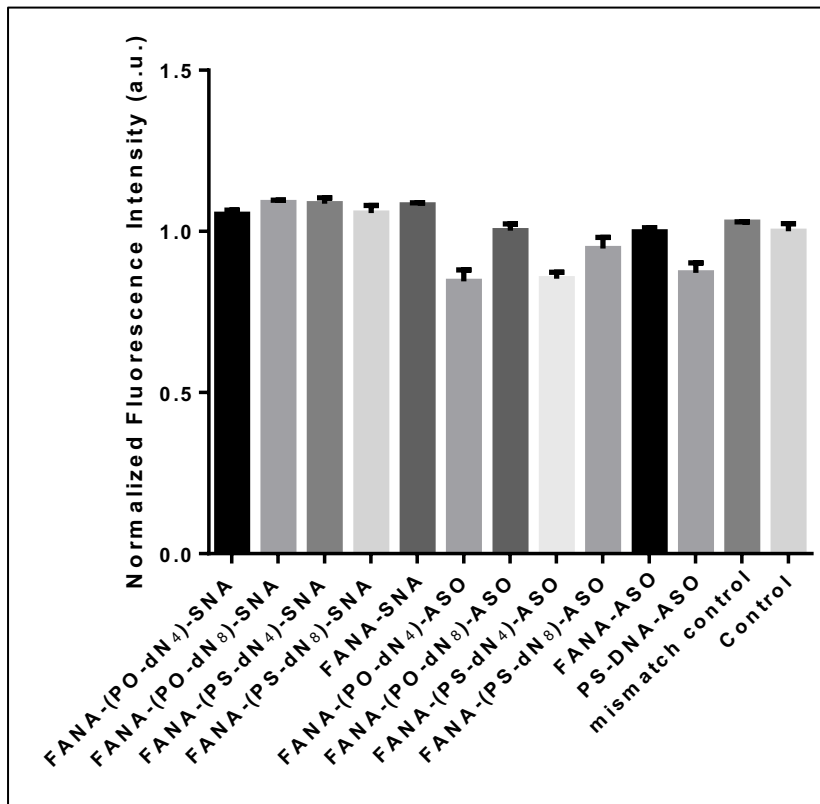
## Serum Stability

Samples (SNA or strands) were prepared in  $1\times$ TAMg buffer. SNA was prepared at  $10 \mu\text{M}$  (in strands) and then diluted with cell culture media (DMEM, 10% FBS, 5% AB/AM) to a concentration of  $2 \mu\text{M}$ . Samples were incubated at  $37^\circ\text{C}$ , and aliquots were taken at different timepoints and frozen until analysis. Aliquots were then treated with proteinase K and resolved on a 15% denaturing (UREA Polyacrylamide) gel to visualize for stability over time. The intensity of the bands was then plotted to visualize the decay over time (x). The equation followed for the plots is:

$$\text{Relative Band intensity}_x = \text{intensity}_x / \text{intensity}_0$$

## VI. Viability assay:

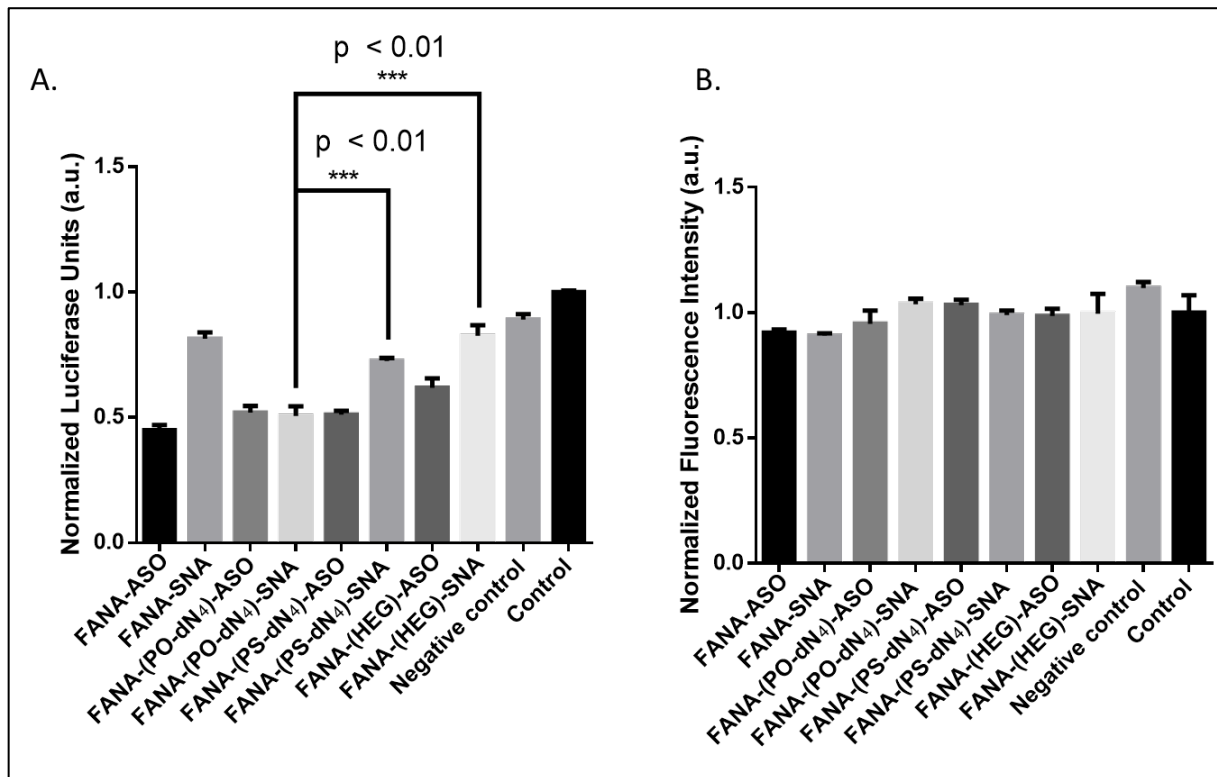
CellTiter-Blue cytotoxicity assay to assess the toxicity of the oligonucleotides used by comparing the metabolic activities of the corresponding cells was done. Results showed no significant toxicity supporting the compatibility and low toxicity of such SNAs and oligos for cell assays. All oligonucleotides were incubated for 24 hours in HeLa cells at a concentration of  $150 \text{ nM}$ , followed by 1.5-hour incubation with MTT reagent (CellTiter-Blue from Promega).



Supplementary Figure 1 Viability assay data for multiple samples in HeLa cells.

## VII. Testing Hexaethylene glycol as a spacer

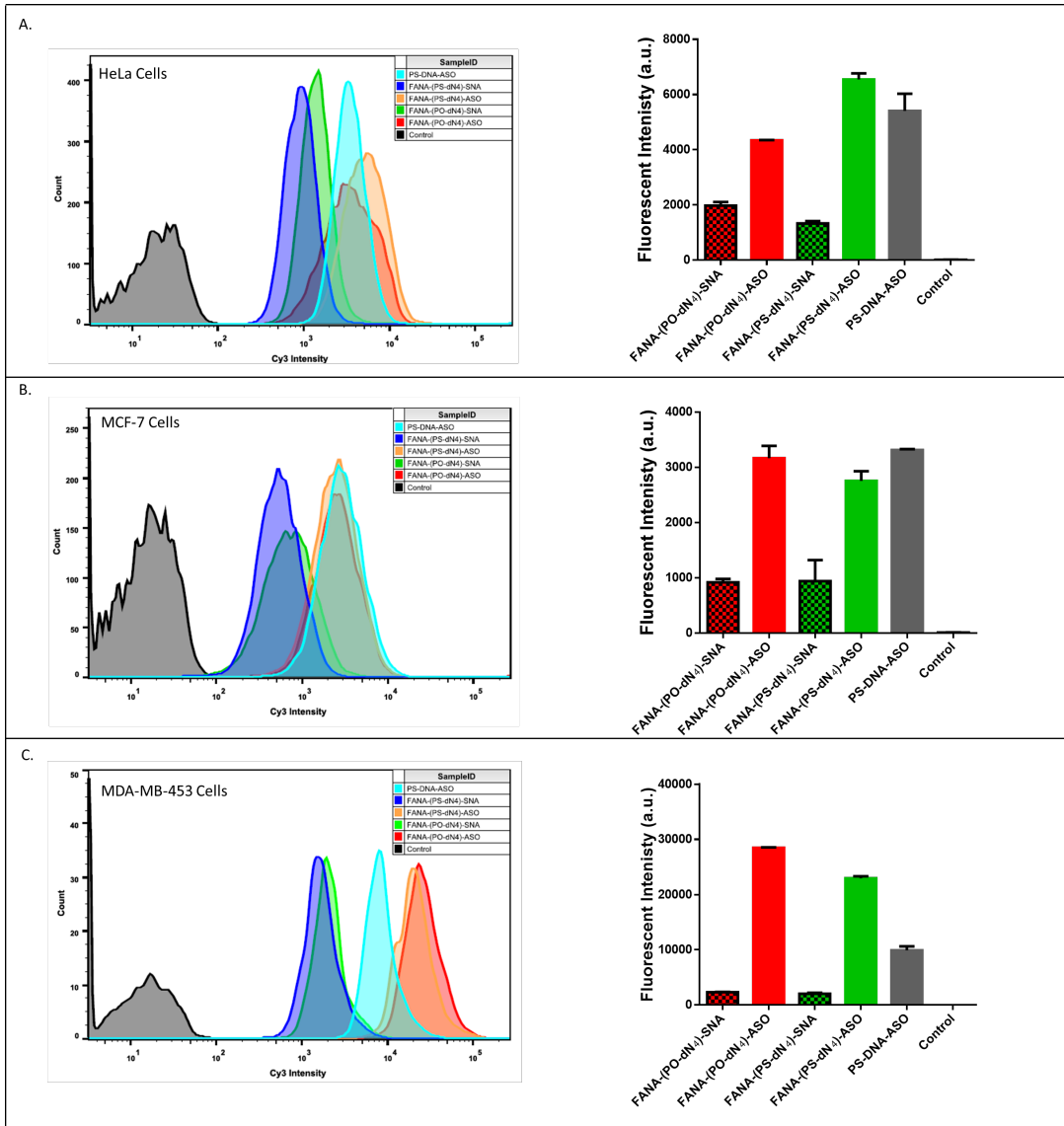
Hexaethylene glycol spacer was used instead of a 4-nucleotide spacer, as another comparison of metabolically non-cleavable linkers. Same experimental conditions were carried out, and the results showed that HEG-spacer SNAs have the same reduced activity compared to mod- (PO-N<sub>4</sub>)-SNA.



Supplementary Figure 2 A) Firefly luciferase knockdown activity comparison between various samples normalized to B) MTT results and AON-free control. Error bars represent SD for triplets for each sample.

## VIII. Flow cytometry uptake assessment of SNAs with different spacers:

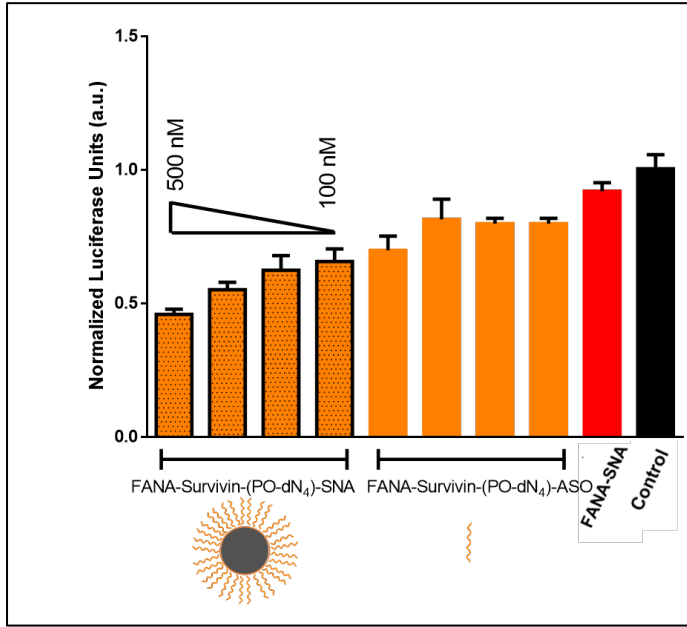
To assess if the increased activity of FANA-(PO-dN<sub>4</sub>)-SNA is coming from its cleavability or from higher uptake into cells compared to FANA-(PS-dN<sub>4</sub>)-SNA, we incubated synthesized both versions with a Cy3 dye (Between the linker and the hydrophobic conjugate) to assess via flow cytometry. Samples were incubated at 0.5  $\mu$ M for 24 hours, followed by live flow cytometry assessment for uptake. The experiment was done in three different cell lines: Hela X1/5, MCF-7 and MDA-453 to showcase the uptake of the SNAs in a variety of cell lines. The results show that they both go into cells to the same extent, further supporting the notion that the higher activity is not coming from increased uptake, but rather from its metabolically cleavable nature that aids its release from endosomes.



Supplementary Figure 3. Flow cytometry experiment to assess the uptake of various samples in different cell lines. Cells were incubated at 0.5  $\mu$ M final concentration of sample for 24 hours, followed by detachment and assessment. Samples were made in duplicates. 10,000 cells count was collected for all samples in HeLa and MCF-7 cells, and 1,000 cells count was collected in MDA-453 cells.

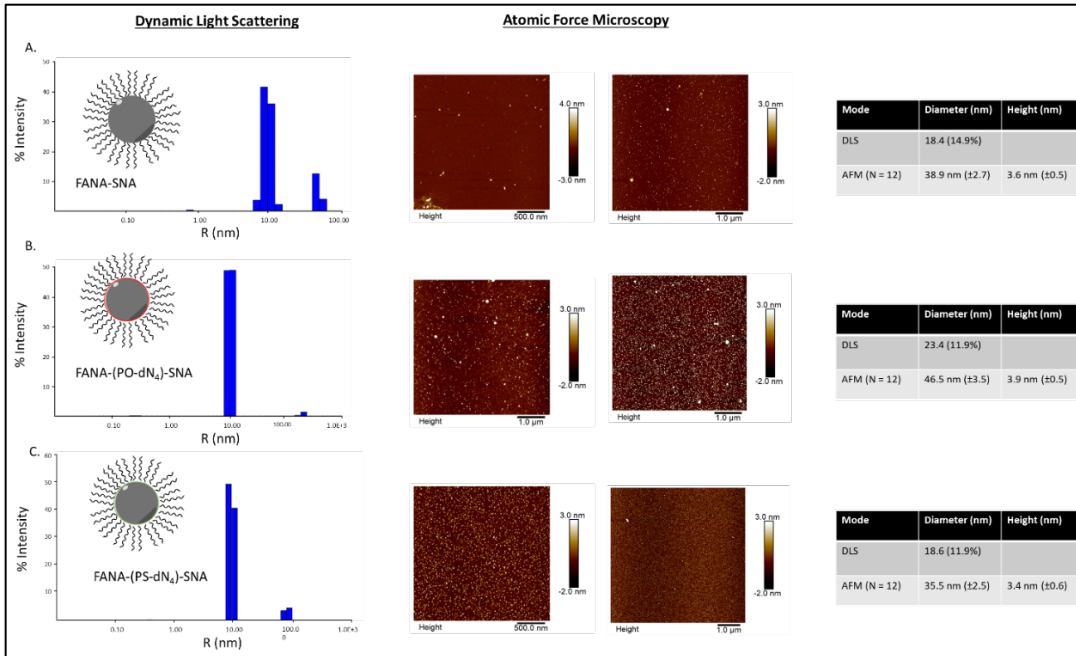


**IX. Titration with various concentration of modSurvivin-SNA**



Supplementary Figure 4 CellTiter-Blue cytotoxicity assay at various concentrations of NAT. metabolic activity was measured after 24 h incubation and normalized to AON-free control. Error bars represent SD for triplets for each sample.

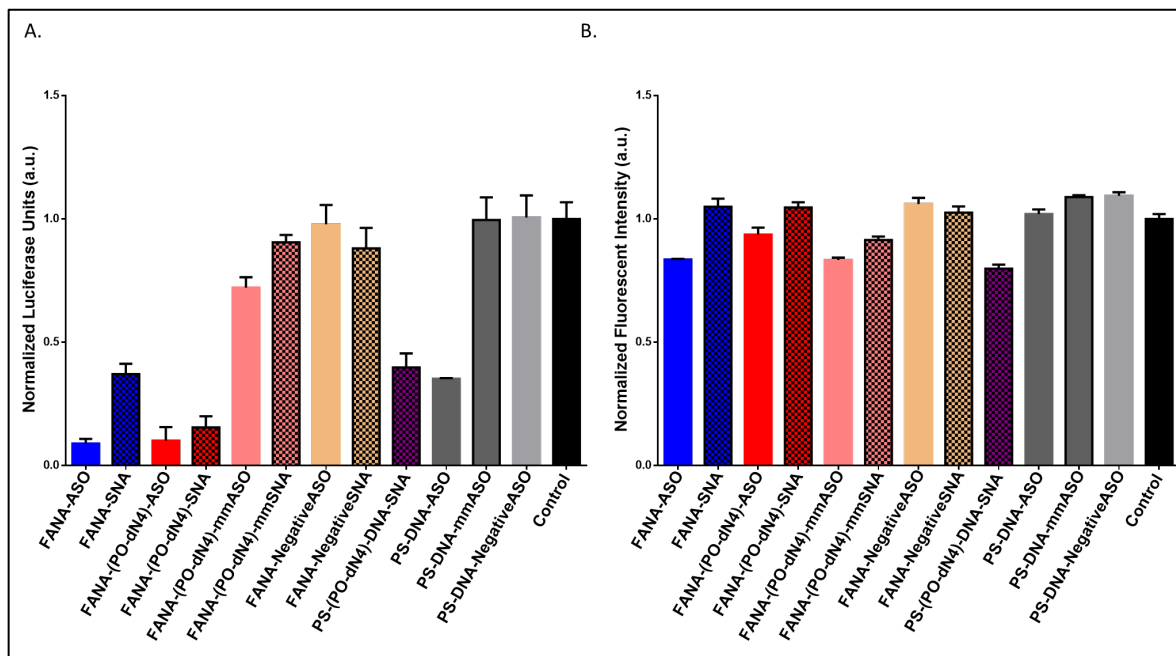
**X. Characterization of FANA-(PS-dN<sub>4</sub>)-SNA:**



Supplementary Figure 5 Dynamic light scattering and additional atomic force microscopy characterization of A) FANA-SNA (no spacer), B) FANA-(PO-dN<sub>4</sub>)-SNA and C) FANA-(PS-dN<sub>4</sub>)-SNA.

## XI. Silencing Controls:

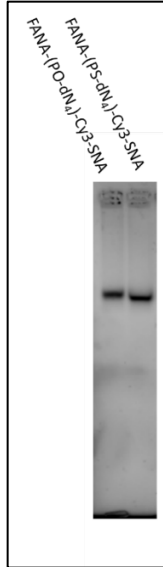
In order to confirm that the silencing activity we are seeing is not due to any cell death or off targeting, we performed a series of controls either as mismatch strands having few mismatched bases, or negative control that has a non-targeting sequence. In both cases, the controls were single stranded ASOs and SNAs, and had FANA modifications. The sequences of each control tested is provided in supplementary table 1.



Supplementary Figure 6 Firefly luciferase knockdown activity comparison between various free FANA-ASO and FANA-SNA. A) Luciferase activity was measured after 24 h incubation and normalized to CellTiter-Blue and Control (no ASO). B) fluorescence measurement of CellTiter-Blue cytotoxicity results normalized to Control (no ASO). Error bars represent SD of triplicate experiments for each sample. ASO final concentration is 150 nM for all samples.

## XII. Agarose Gel of SNA assembly:

the below 2.5% agarose gel shows the assembly and mobility of Cy3 modified FANA-(PO-dN<sub>4</sub>)-SNA and FANA-(PS-dN<sub>4</sub>)-SNA into a tight band with no leftover strands or unconjugated ASO strands.



Supplementary Figure 7 2.5% agarose gel showing the assembly and mobility of Cy3 modified FANA-(PO-dN<sub>4</sub>)-SNA and FANA-(PS-dN<sub>4</sub>)-SNA under cy3 emission. Gel was run at 100V for 1hr45mins in 1xTAMg buffer at 4°C.