Electronic Supplementary Material (ESI) for RSC Advances.

TARGETING HMGA PROTEIN INHIBITS RETINOBLASTOMA CELL PROLIFERATION

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SUPPORTING INFORMATION

1. Experimental Section

1.1 NCLap-HMGA2si conjugate

A. Materials

RB cell, WERI Rb1, was obtained from Riken BioResource center and were cultured in RPMI1640 (Sigma) supplemented with 10% FBS and 1% penicillin streptomycin antibiotics. The cells were maintained at 37°C in 5%CO_2 humidified incubator. The nucleolin aptamer AS1411 was modified with spacer followed by amine group at 5' end and 3' end was tagged with fluorescein (Amine-NCL). HMGA2 siRNA was modified with thiol group at the 5' end and Cytosine and Uracil of sense strand of siRNA with 2' fluoro modification for stability (Thio-HMGA2). Amine-NCL and Thio-HMGA2 were custom synthesized and obtained from Sigma and Dharmacon respectively. The sequence of Amine-NCL aptamer and Thio-HMGA2 siRNA are given in Table 1. Both siRNA and aptamer was reconstituted in double distilled water and stored at -20°C until further use. The following chemicals were purchased and used as such: Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, HCl) from Sigma Aldrich, sulfosuccinimidyl 4-(N-maleimidophenyl)butyrate (Sulfo SMPB) from Thermo Scientific, centrisep spin columns were obtained from Princeton Separations, NJ USA, EZBlue™ Cell Assay Kit from Himedia Laboratories India.

B. Conjugate synthesis

25 µM Amine-NCL aptamer was incubated with 8mM Sulfo SMPB at 37°C for 1 hour. Simultaneously 25 µM of thiol modified siRNA was reduced using 10 mM TCEP in PBS at 37°C for 1 hour. Sulfo SMPB conjugated Amine-NCL and reduced Thio-HMGA2 siRNA were purified using centrisep spin columns and were mixed together and incubated at 37°C for 2 hours. The NCLap-HMGA2si conjugate was then frozen and electrophoresed onto 18% non-denaturing poly acrylamide gel. This conjugate was electrophoresed on 10% non-denaturing poly acrylamide gel and conjugate band was excised and subjected to modified crush and soak method for purification. The gel was cut and crushed into fine particles. 0.1M sodium acetate (pH 6.0) was added and incubated in boiling water bath for 5min followed by incubation at -80°C for 5min. It was then thawed and centrifuged at 3000 g for 5min to remove the gel particles. The supernatant collected was quantified using nano drop spectrophotometer and purified using centrisep spin columns. The purified NCLap-HMGA2si conjugate was concentrated and the molar concentration was determined based on the molecular weight of HMGA2 siRNA.

C. Characterization

The NCLap-HMGA2si conjugate was characterized by non-denaturing polyacrylamide gel electrophoresis (PAGE). 18% non-denaturing PAGE gel loaded with Amine-NCL aptamer, Thio-HMGA2 siRNA and NCLap-HMGA2si conjugate was stained with ethidium bromide. This gel was then imaged in Bio-Rad chemidoc instrument in fluorescein and ethidium bromide channels. Individual channel and also the merged images were analyzed for the presence of respective bands.

D. In vitro stability

The stability of NCLap-HMGA2si conjugate was monitored for a period of 72 hours in 1x PBS and 10% FBS. 10 µl of the conjugate was incubated in 1x PBS and 10% FBS. The mixture was incubated at 37°C and the samples were removed and stored at 4°C after 0, 24, 48 and 72 hour time interval. After 72 hours all the conjugates were loaded onto 18% non-denaturing PAGE gel and the stability of the conjugates were observed. The intensities of the bands were calculated using Image lab 5.2.1 software and normalized to 0 hour.

E. Cellular Internalization Studies

In vitro cellular internalization on WERI-Rb1 cells was performed using Zeiss Axio vision microscope. WERI-Rb1 cells were incubated with 50 nM of the Amine-NCL aptamer and NCLap-HMGA2si conjugate in 1X PBS for 2 hours at room temperature. After incubation the cells were washed twice with 1X PBS to remove the unbound aptamer and conjugate and permeabilized with 4% para formaldehyde for 10 min at room temperature. The cells were then washed twice with 1X PBS and stained with DAPI for 10 min. The cells were finally washed twice with 1X PBS and mounted on slide with anti-fade mountant. The images were acquired in Zeiss Axio vision microscope at 100X magnification.

F. Cytotoxicity assay

In vitro cytotoxicity assay was carried out in WERI-Rb1 cells. 8 x 10^3 WERI Rb1 cells were plated in PLL coated 96 well plates 24 hours before treatment. Amine-NCL aptamer, Thio-HMGA2 siRNA and NCLap-HMGA2si conjugate were used for treatments at the concentrations of 50 and 100 nM. The cytotoxicity of the conjugate was studied by either transfection of Thio-HMGA2 siRNA or direct addition of Amine-NCL aptamer and NCLap-HMGA2si conjugate in 100 µl of incomplete media. Four hours post transfection 100 µl of complete media was added and incubated at 37°C. After 48 hours 100 µl of the media was removed and 10 µl of EZ blue reagent was added to each well and incubated at 37°C for four hours. OD was measured at 580 and 600 nm to calculate the cell viability. All experiments were performed in triplicates.

1.2 NCLAb-HMGAap conjugate

A. Materials

WERI Rb1 cell was purchased from ATCC and were cultured and maintained in custom RPMI media (Cat. A10491, Thermo Fisher Scientific) with 10% heat-inactivated FBS 0.1% ciprofloxacin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 4.5% dextrose at 37 °C in a 5% CO_2-humidified incubator. DNA HMGA aptamer (HMGA AT rich) was custom synthesized and obtained from Integrated DNA technologies, USA. The aptamer was modified with phosphorothioate modifications throughout the length of the oligonucleotide for imparting resistance against nucleases. The 5' end had an amine modification for conjugation purpose and the 3' end had a Cy5 modification with spacer for visualization after cellular uptake. The sequence of HMGA aptamer is given in ESI-Table 1. HMGA aptamer obtained was re-constituted in 1X PBS with pH 7.4 or 1X TAE buffer with pH 8.0, as per manufacturer's instructions. The reconstituted aptamers were aliquoted and stored at -20^{o}C until further use. Anti-Nucleolin antibody (Cat. Ab22758) was obtained from Abcam, USA. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and Sulfo-NHS were obtained from Sigma-Aldrich, USA and were used as received.

B. Conjugate synthesis

The *anti*-nucleolin antibody-HMGA aptamer conjugate (NCLAb-HMGAap) was synthesized by conventional EDC/NHS procedures. (Hermanson, Greg T. 2013 Bioconjugate Techniques, 3rd Edition, Academic Press, London, pp 560.) Briefly, to the nucleolin antibody (20 µg, 0.4 µM) was added EDC (0.4 mM) and Sulfo-NHS (0.62 mM) in 0.1M MES buffer at pH 4.5 and incubated at room temperature for 3 hrs. Excess of EDC and Sulfo-NHS were removed using zeba spin desalting columns (Cat. 89882, Thermo Scientific). The HMGA aptamer (2.5 µg, 0.58 µM) was suspended in 1X PBS at pH 7.4 and to this solution, activated antibody was added and incubated overnight at 4^{o}C. Finally, the conjugate was purified by passing through Amicon centrifuge filters (MWCO 30kDa) to remove excess of unreacted aptamer. The conjugate was washed 4 times and 1% BSA was added as a stabilizer. The conjugate was stored at 4°C until further use.

C. Characterization

UV spectrum profile of NCLAb-HMGAap conjugate was recorded on Cytation 3 (Biotek) microplate reader. The surface charge (zeta potential) of the conjugate was measured by dynamic laser scattering method using a Zetasizer Nano ZS90 (Malvern Instruments Ltd. USA).

For recording charge measurements, the 5 µl of sample was dispersed in 700 µl DI water and analyzed using disposable folded capillary zeta cell with gold electrodes.

D. In vitro stability

The stability of NCLAb-HMGAap conjugate was monitored for a period of 48 hours in 10x PBS and 100% FBS. The conjugates (10 µl) were incubated with 10x PBS or 100% FBS at 0, 24, and 48 hour time interval. After each time interval, the conjugate was analyzed by UV and fluorescence spectroscopy.

E. Cellular Internalization Studies

In vitro cellular internalization on WERI-Rb1 cells was performed using Leica DM5500 B microscope using 10X and 20X objectives. The imaging of nuclear staining and Cy5 labelled aptamer was performed using DAPI LED and Cy5 LED cube at excitation of 648 nm and emission of 670 nm. The WERI-Rb1 cells were seeded at a density of 5 x 10^5 cells/ml in a 6 well plate in custom RPMI medium. The cells were either transfected with HMGAap or added NCLAb-HMGAap conjugate directly and incubated for 24h at 37^{o}C under 5% CO_2. After the incubation period, the cells were centrifuged at 300 g for 5min and washed with 1X PBS twice. The cells were incubated with nuclear staining dye Hoechst 33342 (10 µl of 10 mg/mL) (Cat. H3570, Thermo Scientific) for 15 min at room temperature and washed with 1X PBS. Finally, cells were fixed with 4% paraformaldehyde and mounted on cleaned slide with a coverslip for image analysis. Slides were imaged using a polarized Dark-field fluorescence microscope at 20X magnification. Cell morphology was observed, followed by cellular uptake of the conjugate and images were captured via LAS X Imaging Software on a Leica microscope.

F. Cytotoxicity assay

In vitro cytotoxicity evaluation of conjugates was performed using appropriate controls. The cytotoxicity measurements were performed as per the MTT assay kit protocols (Cat. G4100, Promega). Briefly, WERI-Rb1 cells were seeded at the density of 1×10^5 cells/ml in each well of a flat-bottomed 96-well plate and were incubated at 37°C for 24 hours in a 5% CO_2 environment incubator with adequate humidity. The plate was centrifuged at 300g for 5 min at room temperature and the supernatant was removed. A series of concentrations: 0, 1.25, 2.5, 5, 10 and 20 µg/mL of the conjugate was made in media. Each concentration (100 µl) was added to the plate in pentaplet manner. After 48 hours incubation, 10 µL per well MTT (stock solution 5 mg/mL in PBS) (Promega Corporation, USA) was added and kept for 4 hours, and the formazan crystals so formed were dissolved in 100 µL solubilizing buffer solution. The plates were kept for

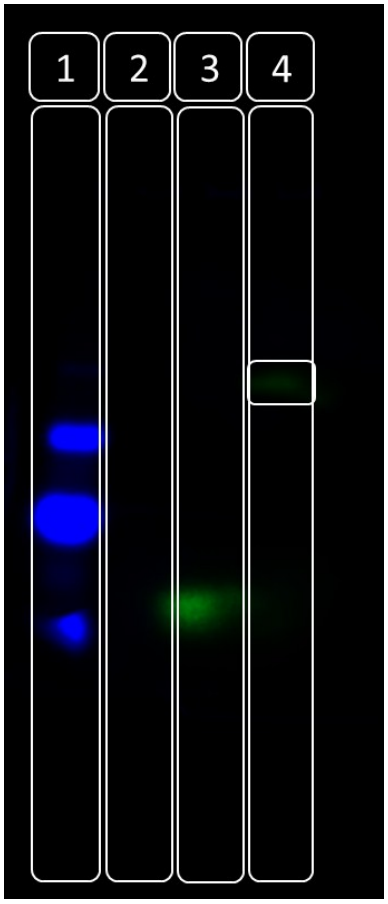
18 hours in dark at 25°C to dissolve all crystals, and the intensity of developed color was measured at 570nm wavelength. Untreated cells were considered 100% viable.

G. Statistical Analysis

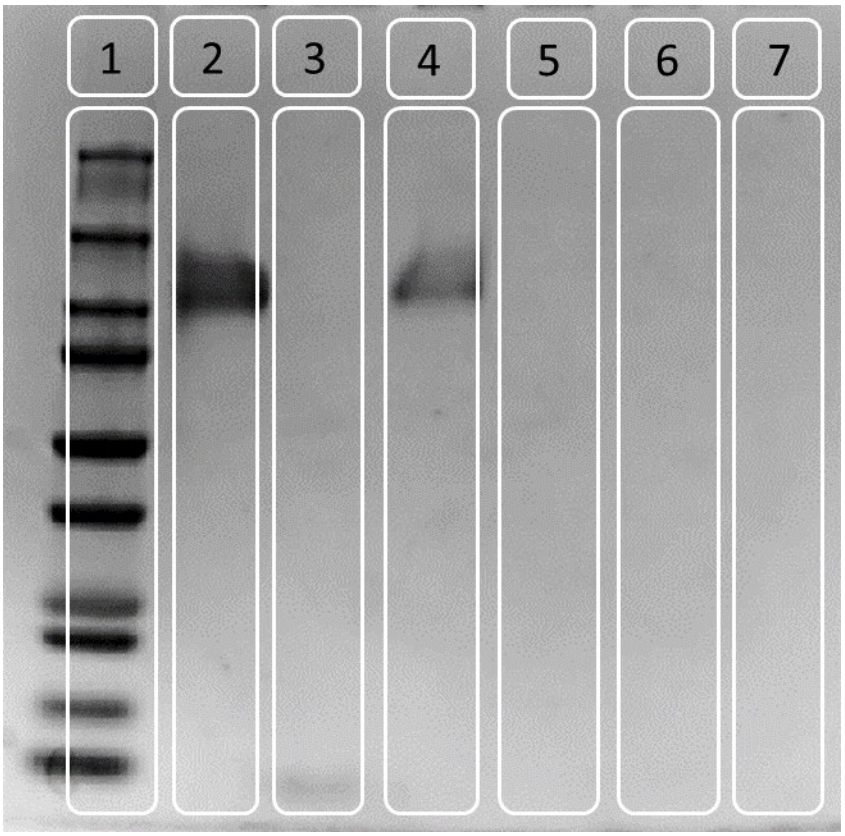
The experiments were carried out in triplicates, and the values were expressed as mean ± SD. One-way ANOVA (non-parametric) using Tukey's multiple comparisons test was used to derive the significance of the test groups. A p value ≤ 0.001 was considered as significant. All the analysis were performed on Prism 7 for Windows using GraphPad Prism Software Version 7.04.

ESI-Table 1: Oligo sequence of amine-NCL aptamer, Thiol-HMGA2 siRNA, and amine modified HMGA aptamer

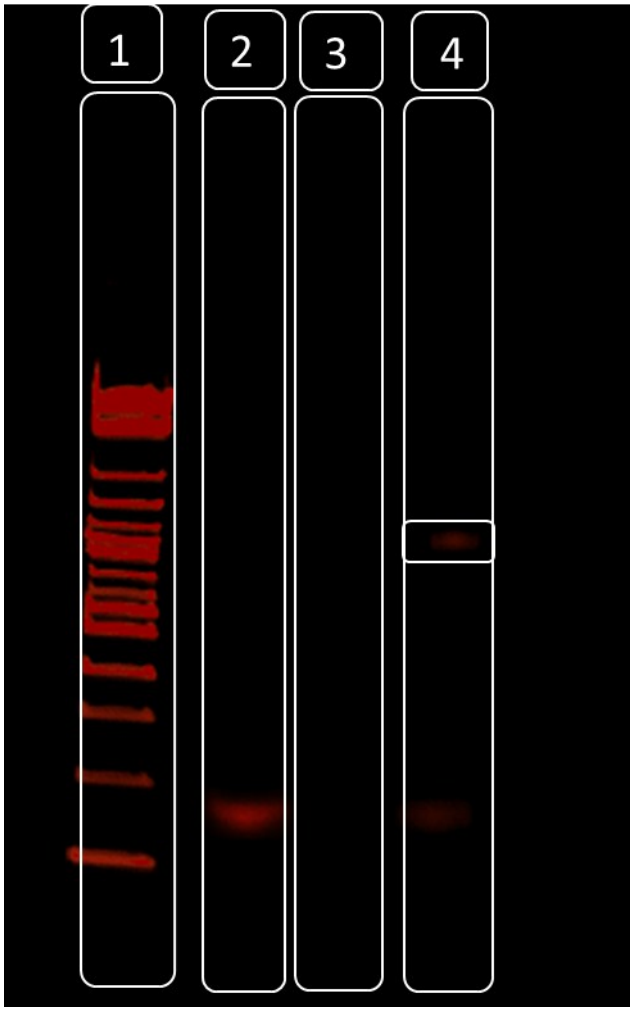
S.No.	Oligo	Sequence
1.	Amine-NCL	(AmC6F)TTTTTTTTTTTTTGGTGGTGGTGGTTGTGGTGGTGGTGG(Flc)
2.	Thiol-HMGA2 siRNA	sense 5' S-S 2'F-CGG2'F-C2'F-CAAGAGG2'F-CAGA2'F-C2'F-C2'F-UAUU 3' anti-sense 5' UAGGUCUGCCUCUUGGCCG UU 3'
3.	Amine modified HMGA aptamer	5' (AmMC6) GGGAAAAAATTTTTTAAAAAACCC 3' (Cy5)



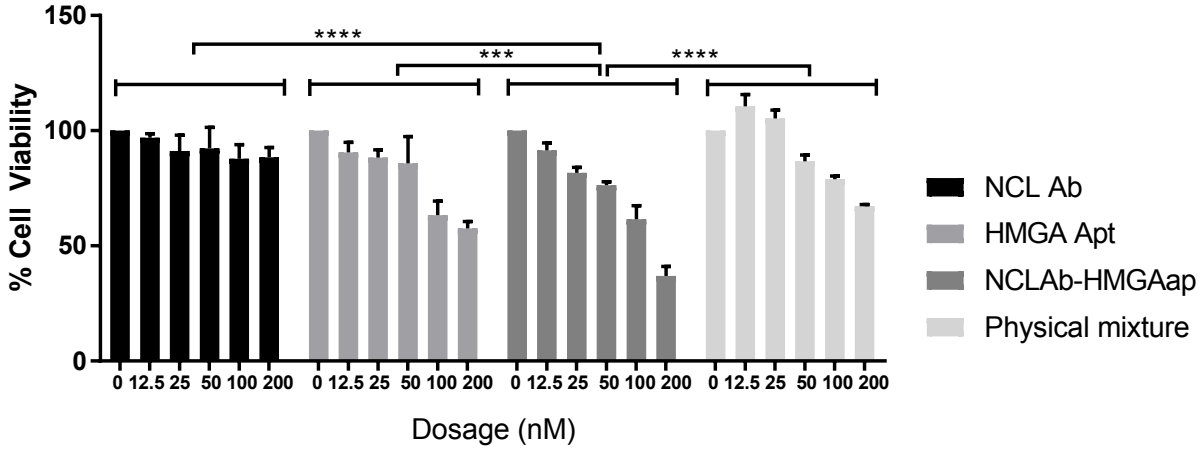
ESI-Figure 1. Fluorescence image of native PAGE gel electrophoresis (Tris Borate EDTA buffer) 1. Protein molecular weight ladder, 2. Free Nucleolin antibody, 3. Free HMGA2-Cy5 Aptamer, 4. Purified Nucleolin Ab-HMGA2 Aptamer conjugate (The conjugate band in lane 4 is highlighted using square inset).



ESI-Figure 2. Coomassie blue stained image of native PAGE gel electrophoresed in Tris glycine SDS running buffer showing the presence of antibody aptamer-Cy5 conjugate. 1. Protein molecular weight ladder, 2. Free Nucleolin antibody, 3. Free HMGA2-Cy5 Aptamer, 4. Nucleolin Ab-HMGA2 Aptamer conjugate, 5. Wash# 1, 6. Wash#2, 7. Wash#3.



ESI-Figure 3. GelRed stained image of native PAGE gel electrophoresed in Tris Borate EDTA buffer showing the presence of antibody aptamer-Cy5 conjugate. 1. DNA base pair ladder, 2. Free HMGA2-Cy5 Aptamer, 3. Free Nucleolin antibody, 4. Nucleolin Ab-HMGA2 Aptamer conjugate.



ESI-Figure 4. Cell Viability assay (MTT) showing treatment of NCLAb-HMGAap conjugate and its individual components, NCL Antibody, HMGA aptamer, physical mixture of NCL ab and HMGA aptamer after 48 hours in RB cancer cells (WERI-Rb-1). Error bars represent the SD of triplicate experiments (n=3) [*** $p < 0.001$, **** $p < 0.0001$].

ESI-Table 2: Quantified amount of NCL antibody present in NCLAb-HMGAap conjugate for each dilution used for cytotoxicity assay

Calculated amount of antibody (nM)	Quantified amount of antibody (nM)
0	0
12	8
25	16
50	32
100	65
200	130