Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Title: Design, Characterization and Biological Evaluation of New Chimeric $4A_{2\text{-}5}$-Antisense Prodrug Combined with Chemotherapy

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Methods and materials

Materials

1 × Phosphate buffer saline (PBS) (pH=7.4) was obtained from Thermo Fisher Scientific. Doxorubicin (DOX) was purchased from Energy Chemical. Fetal bovine serum was provided by EVERY GREEN, ZHEJIANG TIANHANG BIOTECHNOLOGY CO., LTD. DNase I was from Sangon Biotech (Shanghai) Co., Ltd. Acrylamide(cas:97-06-1), N, N'-Methylenebisacrylamide (cas:110-26-9) and Ammonium persulphate (cas: 7727-54-0) were provided by Beijing J&K Scientific Company. Dithiothreitol (DTT) was provided from Beijing InnoChem Science & Technology Co., Ltd.

Oligonucleotides Synthesis and Characterization

Oligonucleotides were all purchased from Suzhou Biosyntech Co., Ltd and Tsingke Biotechnology Co., Ltd. and the molecular weight of them were all determined by MS correctly.

Self-assembly $4A_{2\text{-}5}$-ASON-loop prodrugs

ASONs were dissolved in PBS buffer containing 100 mM NaCl and 10 mM $MgCl_2$. The buffer was heated at 90°C for 5 min, and cooled to room temperature at a rate of 1 °C/min. ASONs in the obtained solution could self-assemble into the conformation of a loop end.

Loading of DOX

A stock solution of DOX (100 μM) was incubated with ASONs (5 μM), in which DOX is about 10-, 5-, 2.5- 2- and 1-fold that of ASONs. 5 μL DOX was fixed and the volume of ASON would be changed accordingly. Then PBS buffer containing 100 mM NaCl and 10 mM $MgCl_2$ was added to make 500 μL. All the samples were incubated overnight at 37°C. The fluorescence emission spectra of ASONs combined with different concentrations of DOX was measured within the excitation wavelength of 490 nm and the emission wavelength range of 510-750 nm.

Measurement of Thermal Transition Curves (T_ms)

ASONs (about 1OD) were added in 1 mL PBS buffer (containing 100 mM NaCl and 10 mM $MgCl_2$) and then tested for the change of absorbance at 260 nm with the temperature rising from 25 to 90 °C or decreasing from 90 to 25 °C (1 °C/min) (Cary-100 Bio UV−visible spectrophotometer, Varian, Palo Alto, CA). The T_m was calculated from the first derivative plots of absorbance vs. temperature.

Circular Dichroism (CD) Assays

ASONs (about 1OD) were added in 200 μL PBS buffer (containing 100 mM NaCl and 10 mM $MgCl_2$) and then measured by a Chirascan Plus CD spectrometer. The temperature was 25 °C, and the wavelength was scanned from 200 to 320 nm.

Protection against Degradation in Serum- and DNase I-Containing Buffer

Fetal bovine serum and DNase I enzyme were used to verify the anti-nuclease degradation of oligonucleotides. The ASONs (about 1OD) were added to the culture medium, including 40% fetal bovine serum or DNase I enzyme. The buffer was incubated at 37°C and sampled at 0, 0.5, 1, 2, 3, 4, 5, 6, and 7 h, separately. A polyacrylamide gel electrophoresis (PAGE) assay and ImageJ software (Broken Symmetry Software) were used for analysis of results.

Stimuli-responsive decomposition of ASONs

ASONs (about 1OD) with a loop structure were added in PBS solution (pH 7.4, supplemented with 100mM NaCl and 10 mM $MgCl_2$) containing DTT (10 mM) and then incubated at 37°C. The samples at corresponding time points were analyzed by PAGE (20%).

The release of DOX

After loaded with DOX as above, ASONs were incubated in PBS solution (pH 7.4, supplemented with 100mM NaCl and 10 mM $MgCl_2$) containing DTT (10 mM) at 37°C, then measured the fluorescence of DOX at corresponding time points.

Reverse-transcriptase quantitative polymerase chain reaction assay

Briefly, MCF-7 cells were seeded in six-well plates (5×10^4 cells/well). After 24 h incubation, corresponding ASONs (0.6 μM) were added and the samples were incubated for another 48 h. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate the total cell RNA, and the PrimeScript RT reagent kit with gDNA eraser (code DRR047A; TaKaRa) was used to synthesize cDNA. SYBR Premix Ex Taq II reagent (code DRR081A; TaKaRa) was used to perform RT-qPCR with the standard cycling program (40 cycles of 95°C for 30 s and 60°C for 5 s). A CFX96 real-time PCR detection system (Bio-Rad Laboratories Ltd., Hercules, CA, USA) was used to obtain melting curves with a temperature increase from 65°C to 95°C at 0.5°C/5 s. The forward and reverse primer sequences for RRM2 are shown in Table S1. The CFX Manage software (version 1.0; Bio-Rad Laboratories Ltd.) was used to perform calculations. The results are expressed as fold changes in expression [2^ (-ΔΔCt)] on a linear scale.

Table S1. RT-qPCR primers.

Gene	Sequence
β-actin	Forward: 5′-CGAGCGCGGCTACAGCTT-3′
	Reverse: 5′-CCTTAATGTCACGCACGATT-3′
RRM2	Forward: 5′-ATGAAAACTTGGTGGAGCGATT-3′
	Reverse: 5′-TGGCAATTTGGAAGCCATAGA-3′

Verification of RNase H and RNase L mediated digestion mechanism

1. RNase H mediated digestion

ASONs were mixed with the equivalent moles of complementary mRNA sequences in 1 × TAE Mg^{2+} buffer (40 mM Tris, 2 mM EDTA·2Na·$2H_2O$, 20 mM acetic acid, 12.5 mM (CH_3COO) 2Mg· $4H_2O$). The water bath was heated to 85°C for 5 minutes and then cooled to room temperature. A concentration of 0 U/mL, 10 U/mL, 50 U/mL, 100 U/mL, 200 U/mL RNase H (#B110070, Sangon Biotech (Shanghai) Co., Ltd.) solution were prepared for use. The above RNase H solution was added to the obtained mixture containing hybrid double chains, which were heated in a water bath at 37°C for 1 h and taken out. The samples were analyzed by PAGE (20%).

ASONs and complementary mRNA sequences were annealed in 1 × TAE Mg^{2+} buffer solution at the molar ratio of 1:10. The annealing process was the same as above. The concentration of 50 U/mL RNase H solution was prepared and added into the annealed solution. The solution was heated in 37°C water bath for 0 h, 1 h, 3 h, 6 h and 18 h.

2. RNase L mediated digestion

The CCK8 method and RT-qPCR were used to verify the function of RNase L. Before administration, cells were pretreated with siRNA (sc-45965, Santa Cruz Biotechnology). Experimental operations were carried out according to the manufacturer's protocol.

***In vitro* Cytotoxicity study**

The cationic lipid reagent (#40802ES02, Shanghai YEASEN Biotech, China) was used for oligonucleotides transfection. It was added with prepared ASONs to MCF-7 cells and incubated for 24 h. Then, the MCF-7 cell growth was detected using the cell counting kit 8 (CCK8) method. A microplate reader (E-max; Molecular Devices, Sunnyvale, CA, USA) was used to measure the optical density of each well at 450 nm.

***In vivo* studies**

Nude BALB/c mice (aged 6-7 weeks, weighing 20 ± 2 g) (Beijing Keyu Animal Breeding Center, Beijing, China) were used for the *in vivo* assessment of the ASONs' antitumor activity. All animal experimental procedures were carried out in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and were also approved by the Animal Care and Use Committee of the Beijing Institute of Pharmacology and Toxicology. All the animals were raised at proper temperatures and had free access to water and standard chow.

MCF-7 cells (1×10^5) suspended in 100 µL PBS were injected subcutaneously into the animals' right flanks. The treatments were begun after the average tumor

volume had reached about 80 mm^3. Intra-tumor injection [0.1 mL (0.3 mmol/L)] were administered daily for 14 days. The mice were sacrificed after the last treatment administration. The tumor volumes [(length × width × height) / 2] were measured with a caliper. The tumors were collected and fixed in 10% formalin overnight, embedded in paraffin, and sectioned at 4 μm thickness. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E).

Supplementary Data

Sigmoidal curves of the T_m values

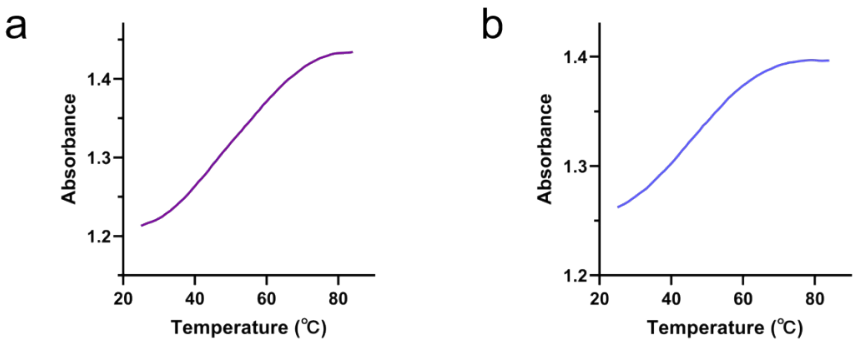

Figure S1. The sigmoidal curves responding to the T_m values of (a) ASON-loop and (b) $4A_{2\text{-}5}$-ASON-loop.

Formation of a hairpin structure.

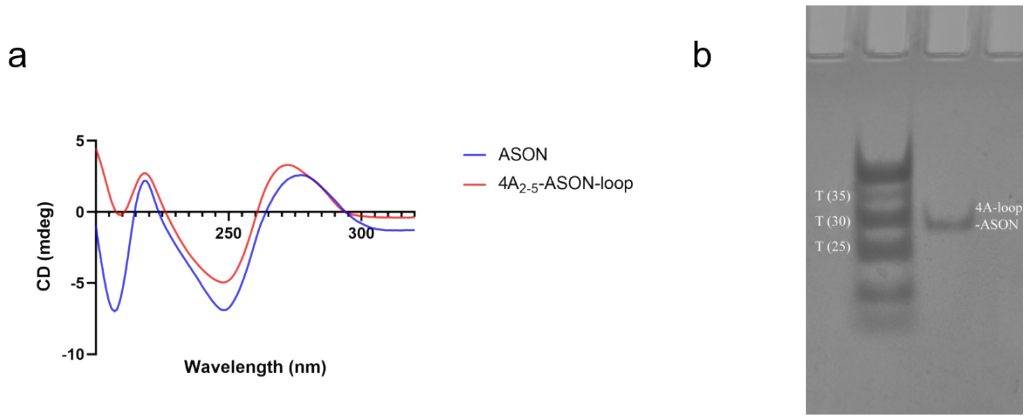

Figure S2. (a) The CD spectra of ASON and $4A_{2\text{-}5}$-ASON-loop. (b) A native-PAGE assay of $4A_{2\text{-}5}$-ASON-loop.

Loading of DOX

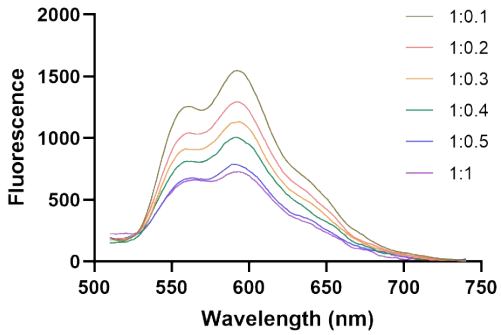

Figure S3. Fluorescence spectra of fixed DOX incubated with different amounts of $4A_{2\text{-}5}$-ASON-loop.

Comparison between ASON/DOX and ASON-loop/DOX

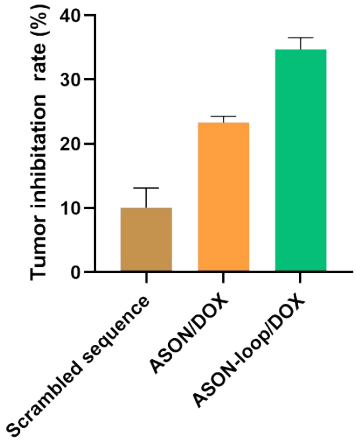

Figure S4. The direct comparison between ASON/DOX and ASON-loop/DOX through a CCK8 assay. The scrambled sequence refers to $(T)_{20}$.

Degradation of mRNA in different concentration RNase H

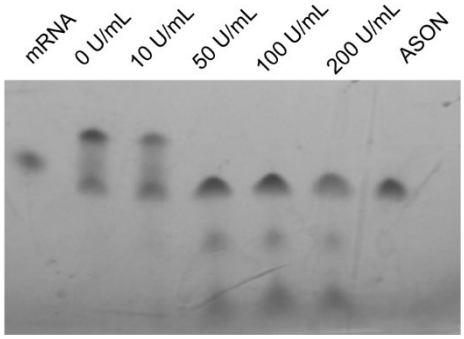

Figure S5. Degradation of mRNA in different concentration RNase H with a molar ratio of 1:1 (ASON: mRNA)

Degradation of mRNA with a molar ratio of 1:10 (ASON: mRNA)

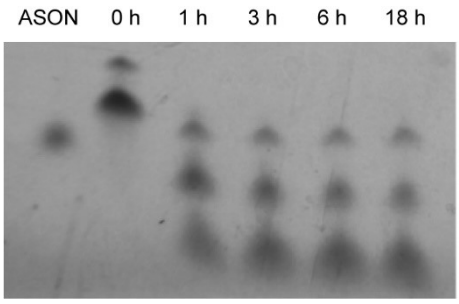

Figure S6. Degradation of mRNA in 50 U/mL RNase H with a molar ratio of 1:10 (ASON: mRNA).

Proliferation inhibition rate of MCF-7 cells with siRNA pre-treated.

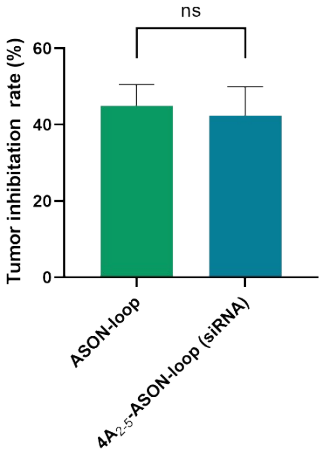

Figure S7. Proliferation inhibition rate of MCF-7 cells through a CCK8 assay with siRNA pretreatment before administration.

HPLC data for ASON, ASON-loop, and $4A_{2\text{-}5}$-ASON-loop.

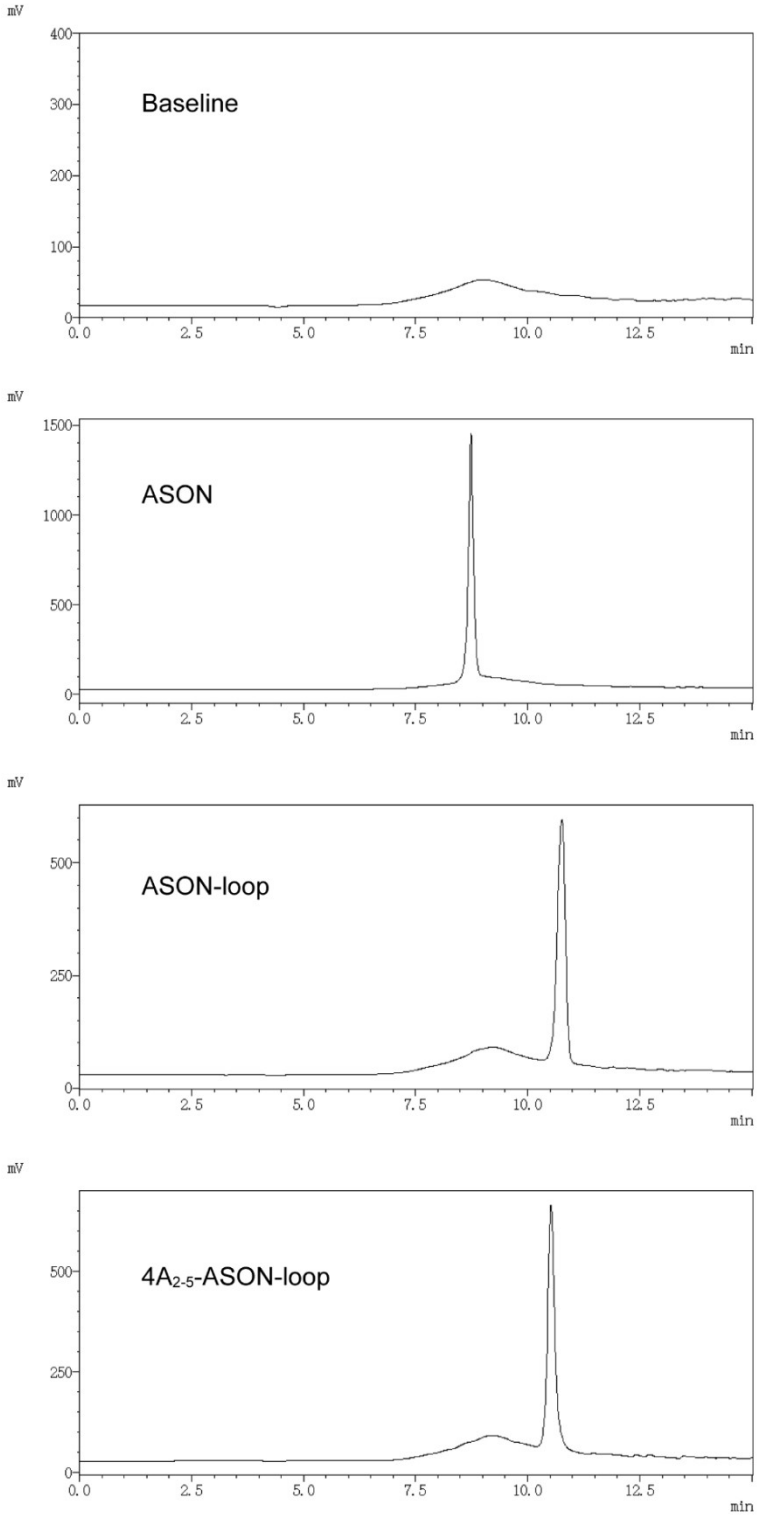

Figure S8. The HPLC spectra of ASON, ASON-loop and $4A_{2\text{-}5}$-ASON-loop. (Phase A: 0.1M acetate/triethylamine buffer containing 5% acetonitrile; phase B: acetonitrile. Flow rate: 1 mL/min. Gradient elution: phase B increased from 5% to 35% within 15 min)