Supplementary Information

Selective inhibition of cancer cell migration using a pH-responsive nucleobase-modified DNA aptamer

Yuyuan Chen,[†] Kunihiko Morihiro,[†,*] Yui Nemoto, Akito Ichimura, Ryosuke Ueki, Shinsuke Sando, and Akimitsu Okamoto*

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Bunkyo-ku, Tokyo 113-8656, Japan
[†]These authors contributed equally to this work.

*To whom correspondence should be addressed. Email: morihiro@chembio.t.u-tokyo.ac.jp (K.M.) or okamoto@chembio.t.u-tokyo.ac.jp (A.O.)

Table of Contents

	Material and methods	S2
Fig. S1	HPLC and MALDI-TOF MS analysis of **CSL1**s	S5
Fig. S2	Melting curves of DNA duplexes	S6
Fig. S3	Flow cytometric analysis of DNA aptamers	S7
Fig. S4	Melting curves of DNA duplexes	S8
Fig. S5	CD spectra of DNA aptamers	S9
Fig. S6	Flow cytometric analysis of DNA aptamers using SNU-1 cells	S10
Fig. S7	Optimization of HGF concentration	S11
Fig. S8	Optimization of **CSL1** concentration	S12
Fig. S9	c-Met inhibition by G-loop	S13
Fig. S10	pH values of cell culture media	S14

Material and methods

General Information. Oligonucleotides were purchased from Hokkaido System Science, Gene Design, and FASMAC. Poly(I:C) was purchased from Tocris Bioscience. A complete list of the DNA and RNA molecules used in this study is provided in Table S1. A549 and MRC-5 cells were purchased from RIKEN BRC CELL BANK (RCB No: 0098 and 0218, respectively). SUIT-2 cells were purchased from the JCRB Cell Bank (JCRB No. 1094).

DNA Synthesis. The synthesis of the **CSL1** aptamers was performed using an automated DNA synthesizer (NTS T-2-TRS; Nihon Techno Service) on a 1.0-μmol scale employing 5-ethylthio-1H-tetrazole (0.25 M in MeCN) as the activator. The concentration of each phosphoramidite was 0.1 M, and the coupling time for modified phosphoramidite was extended to 9 min. The coupling yields were monitored using trityl monitoring. The fully protected **CSL1** aptamers, modified with **An^{C}** linked to the CPG solid support (DMTr-NO), were treated with ammonium hydroxide (28% NH_3 in water) for 2 h at room temperature. The solution was then incubated for 17 h at 55 °C, to remove the protecting group of the base, followed by concentration of the sample. After removal of aqueous ammonia under vacuum, the crude **CSL1** aptamers were roughly purified and detritylated using 2% aqueous TFA on a C18 cartridge (360 mg sorbent, 55–105 μm particle size), and then concentrated. Subsequently, they were freeze-dried using FDM-1000 (JASCO). The resulting **CSL1** aptamers were dissolved in water and carefully purified using reverse-phase (RP)-HPLC. The purified **CSL1** aptamers were quantified by UV absorbance at 260 nm and characterized by MALDI-TOF mass spectrometry using a 3-hydroxypicolinic acid/diammonium hydrogen citrate matrix.

CD Measurement. The CD spectra of the duplexes were recorded using a JASCO J-820 spectropolarimeter. DNA aptamers were dissolved in 10 mM sodium phosphate buffer (pH 7.4) containing 0 or 10 mM KCl, to achieve a final concentration of 2 μM of each oligonucleotide. The samples were boiled at 100 °C, then slowly cooled to room temperature. Spectra were acquired from 320 to 210 nm using a quartz cuvette at room temperature. The scanning speed was set at 50 nm/min, and the response time was 1 s. Molecular ellipticity was calculated using the attached analytical software.

***T_m* Measurement.** The measurements of the melting temperature were performed on a SHIMADZU UV-2550PC spectrophotometer equipped with a TMSPC-8 Tm analytical accessory. Each **CSL1** aptamer was dissolved in PBS (pH 7.4 and pH 6.4), to obtain a final concentration of 2 μM of each strand. An aqueous NaOH solution (1 M) and an aqueous HCl solution (1 M) were used to adjust the pH value of the buffer. The samples were annealed by heating at 100 °C, followed by slow cooling to room temperature. The samples were cooled to 5 °C in the scan cell prior to the T_m measurements. The melting profiles were recorded at 260 nm from 5 to 95 °C at a scan rate of 1 °C/min. The absorbance at 95 °C was set as 1, and the melting curves were normalized. We used the midpoint method to determine the T_m. Melting profiles were collected using spectroscopic experiments, which primarily measure the dependence of absorbance at 260 nm on sample temperature. First, we identify the linear regions before and after the nucleic acid transition, after which the system automatically calculates the formulas for the upper and lower baselines using the midpoint method (y = Ax + B). The fraction of melted base pairs, θ, is then calculated using the standard formula: θ = (A - AL) / (AU - AL), where A, AL, and AU represent the sample absorbance, lower baseline absorbance, and upper baseline absorbance, respectively. When θ = 0.5 and the standard free energy of the transition is zero, the correct definition of the melting temperature, T_m(½), is the temperature at the midpoint of the transition. Although the regions of **CSL1-II** before and after the transition are not as distinct as those of CSL1 and **CSL1-I**, it is still possible to obtain the T_m value using the midpoint method by adjusting the size and position of the regions.

CSL1 (pH 7.4)
lower baseline: y = 0.001238x + 1.115003
upper baseline: y = 0.001190x + 1.241446

CSL1 (pH 6.4)
lower baseline: y = 0.000882x +1.051574
upper baseline: y = 0.000925x + 1.099899

CSL1-I (pH 7.4)
lower baseline: y = 0.001169x + 1.008030
upper baseline: y = 0.001100x + 1.134313
CSL1-I (pH 6.4)
lower baseline: y = 0.001028x + 1.012707
upper baseline: y = 0.000954x + 1.111752

CSL1-II (pH 7.4)
lower baseline: y = 0.001740x + 0.898096
upper baseline: y = 0.001705x + 0.948793
CSL1-II (pH 6.4)
lower baseline: y = 0.001643x + 0.947889
upper baseline: y = 0.001528x + 1.008026

FACS Analysis. A549 cells were seeded in 10-cm dishes, detached using TrypLE, suspended in PBS containing 0.1% BSA at pH 7.4 and pH 6.4, and placed in 1.5-mL tubes. A solution of 1 μM Cy5-**CSL1** was added, to achieve a final concentration of 100 nM, followed by incubation with stirring at 4 °C for 30 min. After two washes with PBS containing 0.1% BSA, the fluorescence intensity of each cell was measured on a Guava® EasyCyte™ Flow Cytometer (Luminex). The results were analyzed using guavasoft 3.3.

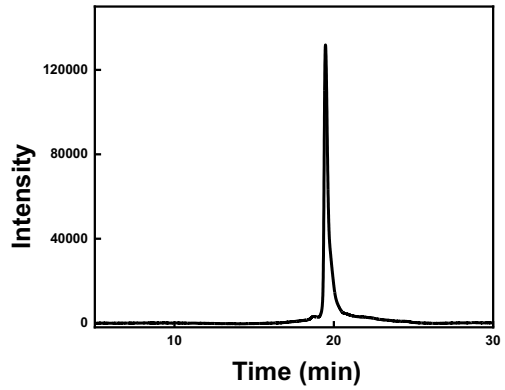
Confocal Laser Scanning Microscopy. A549 cells were seeded onto a 35-mm glass-base dish (using 200 μL of standard growth medium, 1.5×10^5 cells per dish) and allowed to incubate for 24 h. After two washes with PBS, the cells were incubated with Cy5-labeled **CSL1-II** (50 nM) for 15 min in PBS containing 0.5% BSA at pH 7.4 and pH 6.4. Subsequently, the cells were observed using a confocal laser scanning microscope SP-8 (Leica Microsystems). The images were acquired by detecting the fluorescence of Cy5 (Em: 640–670 nm) excited at 638 nm.

ELISA Assay. Cells were passaged into a 12-well tissue culture plate (using 1 mL standard growth medium, 7.0×10^4 cells per well) and incubated for 24 h. After two washes with PBS, A549 cells were incubated with the **CSL1** aptamers (10 nM) for 15 min in PBS containing 0.5% BSA at pH 7.4 and pH 6.4, HGF (250 pM) was added and the cells were incubated for 15 min, then lysed with lysis buffer. The lysates were collected into 1.5-mL tubes and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was collected and stored in the refrigerator at –20 °C. The capture antibody was precoated on a 96-well tissue culture plate (100 μL per well) and incubated for 24 h. After three washes, Blocking One-P was added and the samples were incubated for 2 h at room temperature. After removing the solution, c-Met-secreting cells pretreated with **CSL1** aptamers were incubated in antibody-coated wells for 2 h at room temperature. After five washes, an HRP-conjugated mouse anti-phosphotyrosine antibody was added to the samples, to bind c-Met, for 2 h at room temperature in the dark. Five washes later, a substrate solution was added to catalyze an enzymatic color reaction proportional to the antibody for 20 min at room temperature. Subsequently, stop solution was added to each well and the OD was measured at 450 nm on an BioTek Cytation 5 Imaging Reader.

Wound Healing Assay. An ibidi Culture-Insert 2 Well was attached to each well of two 6-well tissue culture plates. Next, 70 μL of SUIT-2 cells (10×10^5 cells per well) and MRC-5 cells (5×10^5 cells per well) were seeded onto each well and incubated at 37 °C with 5% CO_2 for 24 h. The cell density was verified 24 h later under the microscope. The Culture-Insert 2 Well was then gently removed using sterile tweezers, and the cell layer was washed with PBS to remove cell debris and nonattached cells. After

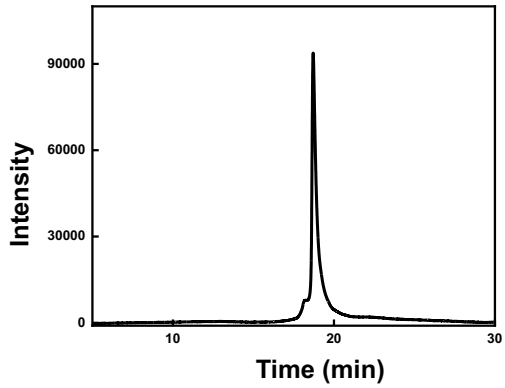
the medium was replaced by cell-free medium, **CSL1-II** (500 nM) and HGF (200 pM) were added by applying the recommended volume of 1 mL. The dish was placed on the microscope and moved until the gap and both cell fronts could be captured in the image using a 4×/5× objective lens. The observation process was initiated by acquiring images several times throughout the following 48 h, at time intervals of 30 min. The area of the blank region before and after the artificial gap between the cells was calculated using Image J.

CSL1-I



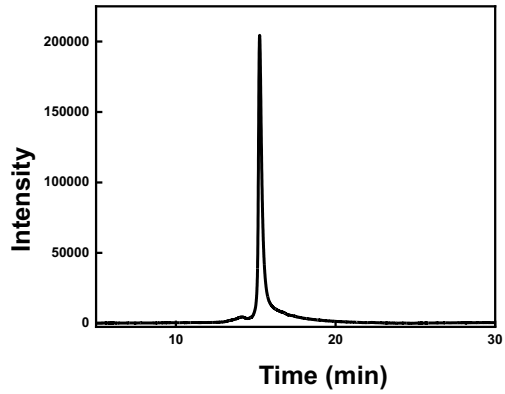
MALDI-TOF MS
Calcd. $[M+H]^+$: 15651
Found $[M+H]^+$: 15649

CSL1-II



MALDI-TOF MS
Calcd. $[M+Na]^+$: 15657
Found $[M+Na]^+$: 15662

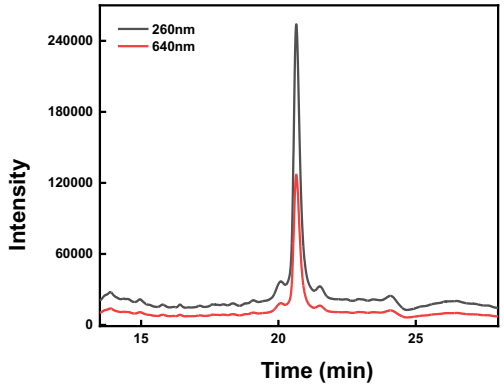
CSL1-III



MALDI-TOF MS
Calcd. $[M+H]^+$: 15619
Found $[M+H]^+$: 15631

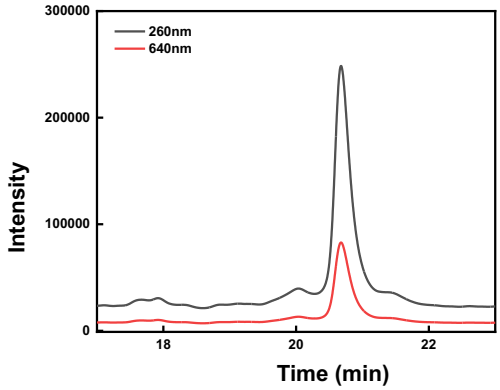
Fig. S1. HPLC and MALDI-TOF MS analysis of **CSL1**s.

Cy5-**CSL1-I**



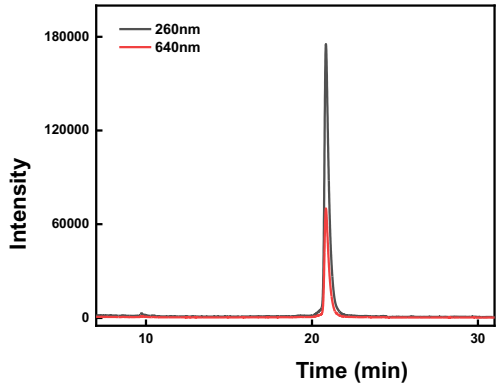
MALDI-TOF MS
Calcd. $[M+H]^+$: 16184
Found $[M+H]^+$: 16195

Cy5-**CSL1-II**



MALDI-TOF MS
Calcd. $[M+Na]^+$: 16191
Found $[M+Na]^+$: 16207

Cy5-**CSL1-III**



MALDI-TOF MS
Calcd. $[M+H]^+$: 16153
Found $[M+H]^+$: 16133

Fig. S2. HPLC and MALDI-TOF MS analysis of Cy5-modified **CSL1**s.

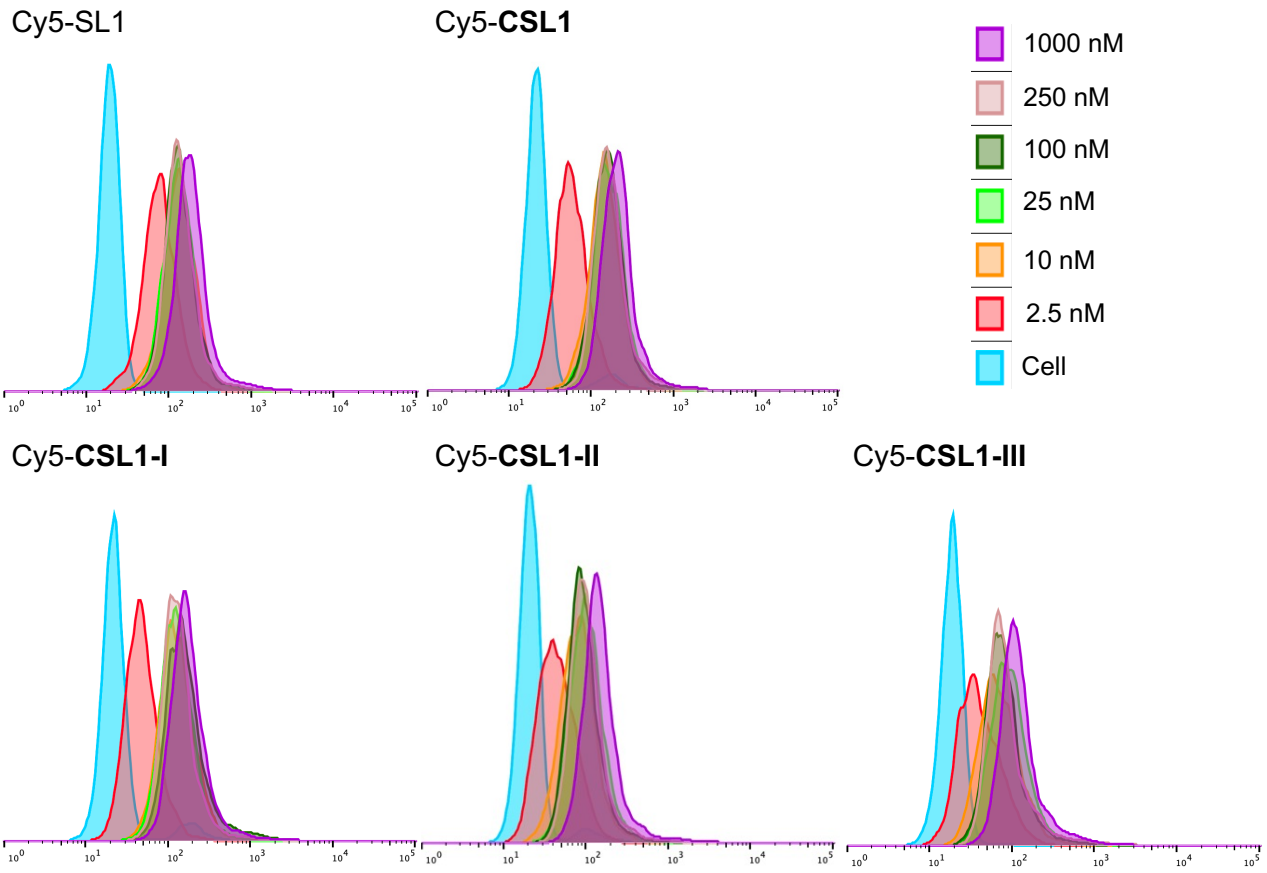


Fig. S3. Flow cytometric analysis of DNA aptamers.

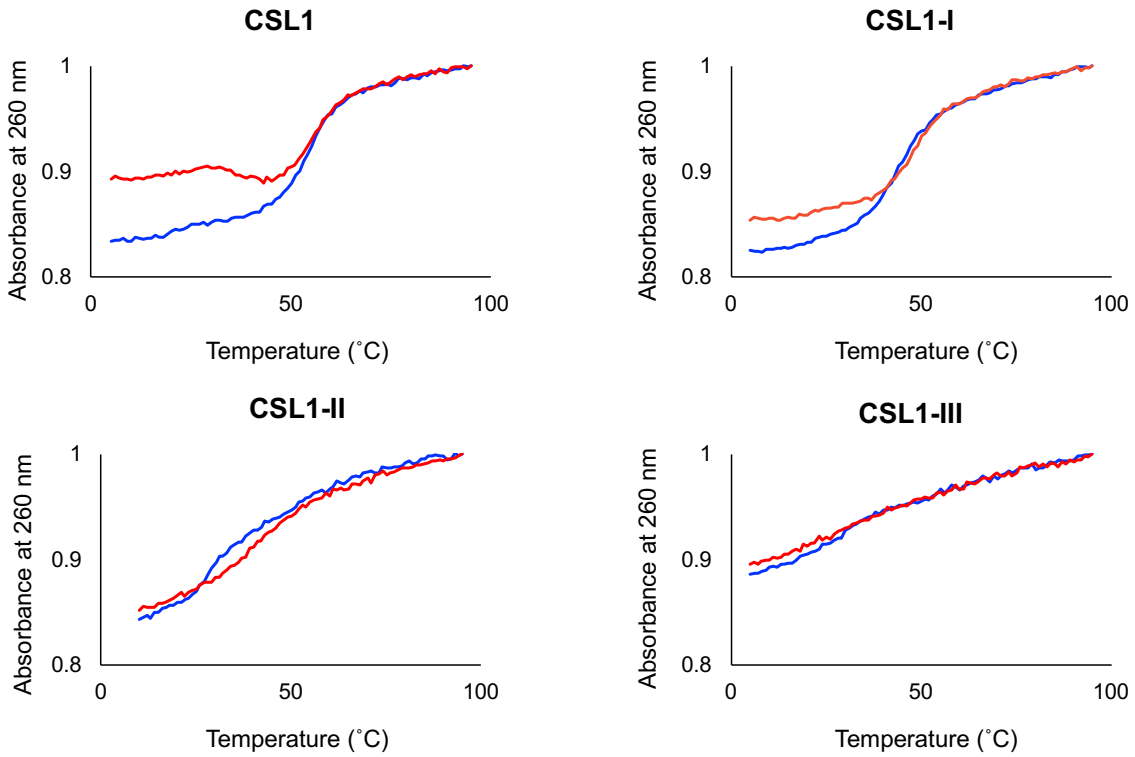


Fig. S4. Melting curves of DNA duplexes. The blue lines indicate under pH 7.4, and the red lines indicate under pH 6.4.

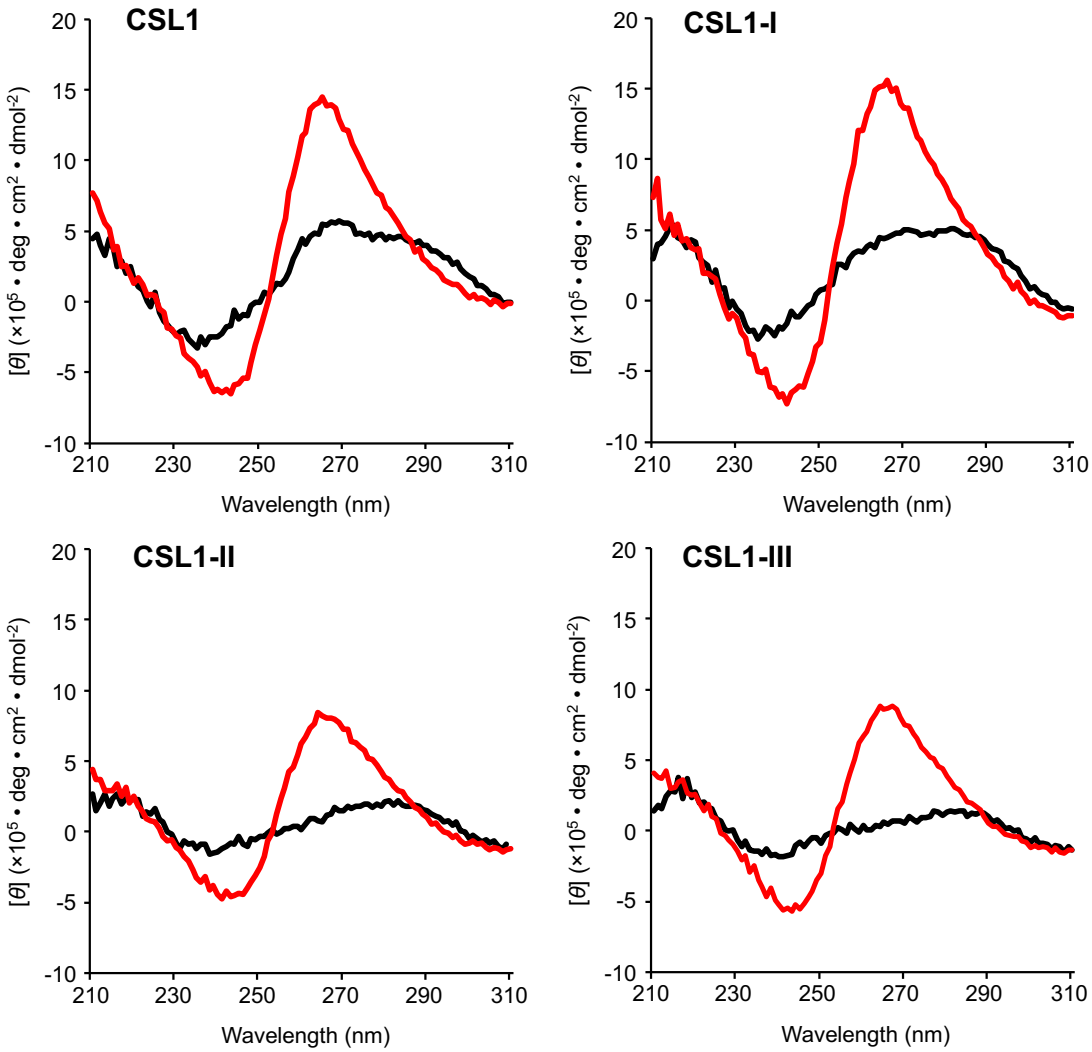


Fig. S5. CD spectra of DNA aptamers. The black lines indicate the absence of KCl, and the red lines indicate the presence of KCl (10 mM).

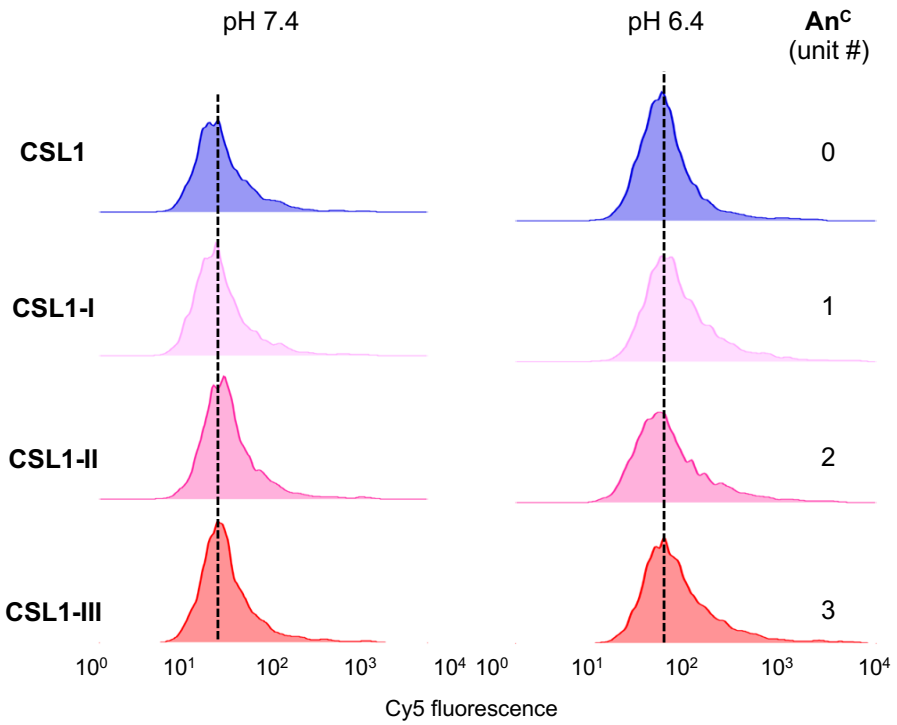


Fig. S6. Flow cytometric analysis of DNA aptamers using SNU-1 cells.

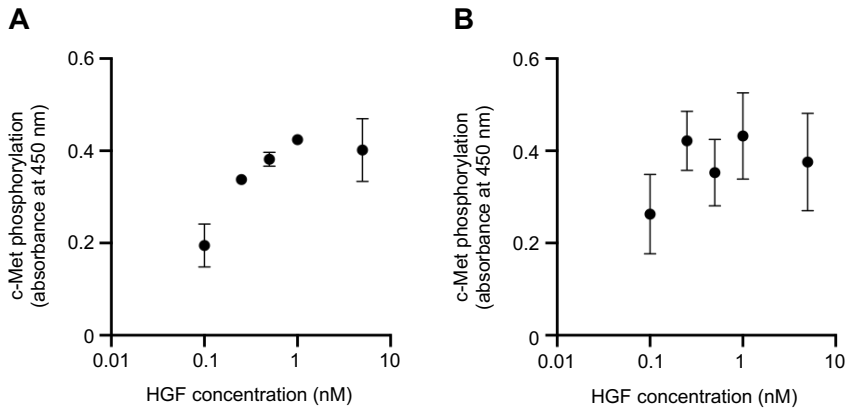


Fig. S7. Optimization of HGF concentration. Three independent experiments were averaged, and the error bars represent standard deviations.

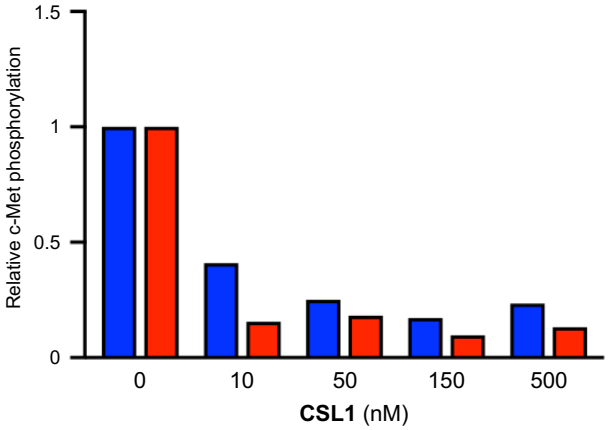


Fig. S8. Optimization of **CSL1** concentration at pH 7.4 (blue) and 6.4 (red).

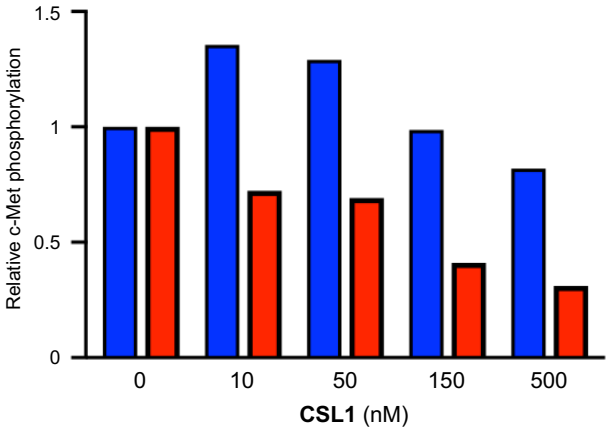


Fig. S9. c-Met inhibition by G-loop at pH 7.4 (blue) and 6.4 (red).

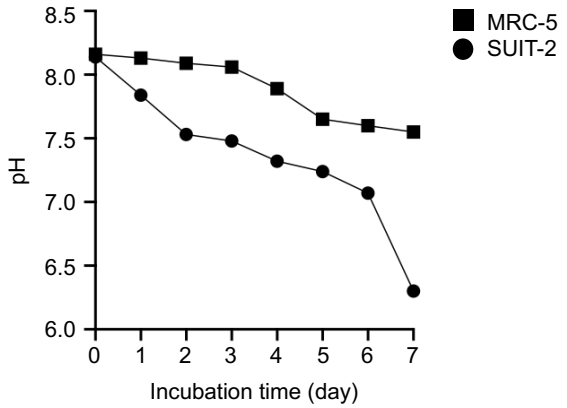


Fig. S10. pH values of cell culture media. SUIT-2 cells (1×10^5 cells per dish) and MRC-5 cells (4×10^5 cells per dish) were seeded onto two 35 mm dishes in 2 mL of standard growth medium, respectively, and incubated at 37 °C with 5% CO_2 for 7 days. Each day, 100 µL of growth medium was collected, and the pH value was measured using a pH meter (LAQUAtwin).