Supporting Information

Circular bivalent aptamers enable in vivo stability and recognition

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EXPERIMENTAL SECTION

Reagents and Materials. Bovine Serum Albumin (BSA) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, 99.9%) were purchased from Beyotime Co. (Jiangsu, China). Magnesium chloride hexahydrate ($MgCl_2$•$6H_2O$, AR), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, AR, 98%), and boric acid (H_3BO_3, AR, 99.5%) were purchased from Aladdin Industrial Co. (Shanghai, China). N, N, N', N' - tetramethylethylenediamine (TEMED), 30% acrylamide, ammonium persulfate (APS, 98%), and agarose were purchased from Beijing Dingguo Changsheng Biotechnology Co. T4 DNA ligase with 10× T4 DNA ligase buffer (660 mM Tris-HCl, pH7.6, containing 66 mM $MgCl_2$, 100 mM DTT and 1 mM ATP) and Exonuclease I (Exo I) with 10× Exo I buffer (670 mM Glycine-KOH, pH 9.5, containing 10 mM DTT and 67 mM $MgCl_2$) were purchased from Takara Biotechnology Co. (Dalian, China). All reagents were received and used without further purification.

Cell lines and cell culture. CCRF-CEM cells and Ramos cells were purchased from ATCC. Cells were cultured in RPMI medium 1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution (PS) at 37 °C in 5% CO_2 atmosphere. Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco) was used to wash cells.

Washing buffer and binding buffer. Washing buffer was prepared with D-PBS supplemented with 4.5 g/L of glucose and 5 mM of $MgCl_2$. Binding buffer was prepared with D-PBS supplemented with 4.5 g/L of glucose, 5 mM of $MgCl_2$, 0.1 mg/mL of yeast tRNA, and 1 mg/mL of BSA.

Construction of circular bivalent aptamers. HPLC-purified DNA library (Lib) and aptamers (Table S1 and Table S2) used in this study were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). To prepare cb-aptamer, its two components were first dissolved in T4 DNA Ligase buffer in appropriate concentration and then heated at 95 °C for 5 min, followed by quick chilling to 16 °C, allowing the formation of circular aptamers with two nicks (nc-aptamers). The nc-aptamers were then incubated with T4 DNA ligase at 16 °C for 12 h to form cb-aptamers. Finally, the solution was incubated at 75 °C for 10 min to denature the ligase. DNA samples were extracted by phenol-chloroform extraction and ethanol precipitation. The concentration of DNA was determined with UV-vis spectrophotometry prior to usage.

Construction of circular trivalent aptamer. To prepare circular trivalent Sgc8 (ct-Sgc8), sequences (No.1, No.2 and to No.3 in Table S2) were used as Y-type building blocks. The three DNA sequences were mixed stoichiometrically in T4 DNA ligase buffer at appropriate concentrations. The mixed solution was annealed by heating at 90 °C for 5 min, followed by cooling slowly to 16 °C. The Y-type DNA was then mixed with annealed sequence No.4 (Table S2) in T4 DNA ligase buffer at 4 °C for 6 h and finally incubated with T4 ligase at 16 °C for another 12 h.

Melting temperature analysis. Melting profiles were acquired and analyzed on an IQ5 multicolor real-time PCR detection system (BIO-RAD). Three μM Sgc8 (No. 1 sequence in Table S3), 1.5 μM nc-Sgc8 (No. 2 and No. 3 sequences in Table S3), 1.5 μM duplex fragment of cb-Sgc8 (No.4 and No.5 sequences in Table S3) and 1.5 μM cb-Sgc8 prepared with No. 2 and No. 3 sequences in Table S3 were incubated in different buffers at 16 °C for 1 h and then slowly heated to 95 °C with a heating rate of 0.5 °C/30 s to acquire a fluorescence signal.

Stability analysis of aptamers in exonuclease solution and serum. Three μM fluorescein amidate (FAM)-labeled aptamer, 1.5 μM nc-aptamers or 1.5 μM cb-aptamers were incubated with exonuclease I (Exo I) or RPMI 1640 with 10% fetal bovine serum (FBS) at 37 °C. At designated time points, samples were heated at 95 °C for 5 min to denature the enzyme and subsequently stored at -20 °C until all samples were collected. Samples were then thawed on ice for electrophoresis or flow cytometry.

Gel electrophoresis assay. Polyacrylamide gel electrophoresis and agarose electrophoresis were used to evaluate the integrity of aptamers. The former was used to analyze sequence integrity after aptamers were treated with 10% FBS. The latter was used to analyze sequence integrity after treatment with Exo I. Ten μL DNA samples were mixed with 2 μL 6× loading buffer and then loaded into 3% agarose gel or 18% polyacrylamide gel in electrophoresis buffer (9 mM Tris, pH 8.0, containing 9 mM boric acid and 1 mM EDTA). After electrophoresis, the gels were analyzed with a molecular imager (BIO-RAD).

Sample preparation for flow cytometry and confocal microscopy. A total of 4×10^5 cells (CCRF-CEM or Ramos cells) were washed with washing buffer via centrifugation at 1000 rpm and then incubated with FAM-labeled aptamers or ssDNA library in 200 μL of binding buffer at 4 °C or 37 °C for 1 h for binding assay. After washing twice with washing buffer (4 °C or 37 °C), the samples were collected and then resuspended in binding buffer for flow cytometry and confocal microscopy. Flow cytometry was performed with a BD FACSVerse™ system. Confocal microscopy was carried out on a FV1000-X81 confocal microscope (Olympus).

To prepare samples for endocytosis analysis, the cells were treated with trypsin for 3 min at 37 °C after incubation with FAM-labeled aptamers or library for 4 h at 37 °C. Then cells were washed twice with 400 μL of DPBS. Finally, cell samples were suspended in 500 μL of DPBS for flow cytometric analysis.

In Vivo Fluorescence Imaging. Four- to six-week-old BALB/c nude mice (Shanghai SLAC Laboratory Animal Co., Ltd.) received a subcutaneous injection of 1×10^7 CCRF-CEM cells at the right axilla. Tumors were then allowed to grow over a period of 21 to 28 days until tumor volume reached ~300 mm^3.

Tumor-bearing BALB/c nude mice were anesthetized to be motionless with both tranquilizer and anesthetic before 4.5 nmol of Cy5-labeled cb-Sgc8, Sgc8 or library was injected intravenously via the tail vein. At the designated time points, fluorescence images of live mice were collected by an IVIS Lumina II in vivo imaging system (Caliper LifeSicence, USA).

Table S1. Circular bivalent aptamers (cb-aptamers) and their components

Name	No.	Strand components (5' – 3')
cb-Sgc8 with additional 9-base pair	1	*P*-**CGT AAA TCA** GTC TAA CTG CGC CGC CGG GAA AAT ACT GTA CGG T T(FAM) A GAC
	2	*P*-**TGA TTT ACG** GTC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG T T(FAM) A GAC
cb-Sgc8 with additional 13-base pair	3	*P*-**CGT AAA TCA GTC A**GT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GT T(FAM) AGA C
	4	*P*-**TGA CTG ATT TAC G**GT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GT T(FAM) AGA C
cb-Sgc8 with additional 17-base pair	5	*P*-**CGT AAA TCA GTC ACT AG**G TCT AAC TGC TGC GCC GCC GGG AAA ATA CTG TAC GGT T(FAM) AG AC
	6	*P*-**CTA GTG ACT GAT TTA CG**G TCT AAC TGC TGC GCC GCC GGG AAA ATA CTG TAC GGT T(FAM)AG AC
cb-TD05 with additional 13-base pair	7	*P*-**CGT AAA TCA GTC A**AGG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC C T(FAM) C CCT
	8	*P*-**TGA CTG ATT TAC G**AGG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC C T(FAM) C CCT
cb-XQ-2d with additional 13-base pair	9	*P*-**CGT AAA TCA GTC A**GC TCA TAG GGT TAG GGG CTG CTG GCC AGA TAC TCA GAT GGT AGG GTT ACT A T(FAM) G AGC
	10	*P*-**TGA CTG ATT TAC G**GC TCA TAG GGT TAG GGG CTG CTG GCC AGA TAC TCA GAT GGT AGG GTT ACT A T(FAM) G AGC
Library (Lib)		FAM-CTA ACT GAN NNN NNN NNN NNN NNN NNN NNN NCG GTT AGA

Table S2. Circular trivalent Sgc8 (ct-Sgc8) and its components

Name	No.	Stand components (5' - 3')
ct-Sgc8	1	*P*-**CGT AAA TCA GTC A**AC CTG TCT GCC TAA TGT GCG TCG TAA G
	2	*P*-**CGT AAA TCA GTC A**AC TTA CGA CGC ACA AGG AGA TCA TGA G
	3	*P*-**CGT AAA TCA GTC A**AC TCA TGA TCT CCT TTA GGC AGA CAG G
	4	*P*- **TGA CTG ATT TAC G**GT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT AGA C

Table S3. Aptamers and DNA sequences for thermal melting analysis and their components

Name	No	Strand components (5' – 3')
Sgc8	1	*P*-**TGA CTG ATT TAC G**GT CT**(Dabcyl)**A ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT(FAM) AGA C
cb-Sgc8	2	*P*-**CGT AAA TCA GTC A**GT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT AGA C
	3	*P*-**TGA CTG ATT TAC G**GT CT**(Dabcyl)**A ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT(FAM) AGA C
Duplex fragment of cb-Sgc8	4	**Dabcyl-**CGG TTA GAC CGT AAA TCA GTC AGT CTA ACT G
	5	CGG TTA GAC TGA CTG ATT TAC GGT CTA ACT G-FAM

Table S4. Melting temperatures (Tm) of aptamers and the duplex fragment of cb-Sgc8 in different buffer solutions

DNA	T_m in buffer 1 / °C	T_m in buffer 2 / °C	T_m in buffer 3 / °C
Sgc8	49.8 ± 0.6	40.4 ± 1.1	46.8 ± 0.6
nc-Sgc8	51.9 ± 0.5	42.3 ± 0.4	46.6 ± 0.2
cb-Sgc8	81.3 ± 0.3	77.7 ± 0.4	80.1 ± 0.2
Duplex fragment of cb-Sgc8	69.1 ± 0.2	67.0 ± 0.7	65.4 ±0.5

Note: Buffer 1: 66 mM Tris-HCl, 6.6 mM Mg^{2+}, pH 7.6
Buffer 2: D-PBS plus 0.66 mM Mg^{2+}, pH 7.4
Buffer 3: 6.6 mM Tris-HCl, 0.66 mM Mg^{2+}, pH 7.6

Table S5. Chemical modified aptamer and its components

Name	Stand components (5’ - 3’)
Inverted dT Sgc8	ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TT(FAM)A GA***T(inverted)***
Phosphorothi -oate Sgc8	***A*T*C *T*A**A CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG T**T(FAM)*A* G*A*T***
2’-O-methyl Sgc8	**A**T**C** T**A**A CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG T T(FAM)**A GA**T

Note: * and underlined letter represent phosphorothioate site and 2’-O-methyl oligoribonucleotide, respectively.

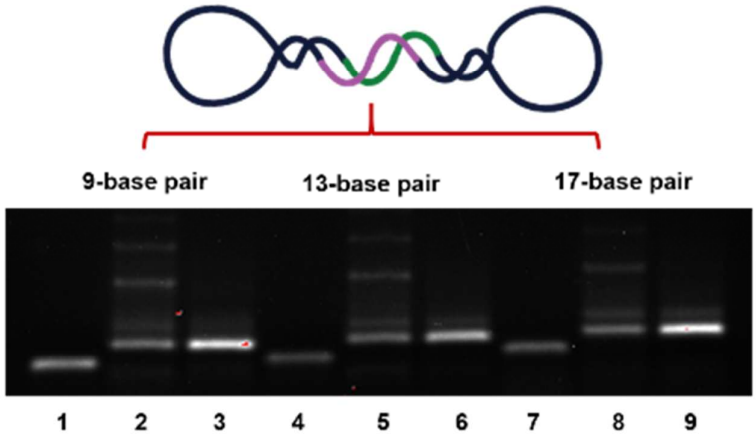

Figure S1. The effect of annealing conditions of denatured aptamers on the formation of cb-Sgc8 with additional 9-base, 13-base or 17-base complementary sequences, as determined by agarose electrophoresis. Lanes 1, 4 and 7 represent sequence No.1, No.3 and No.5 (Table S1), respectively. Lanes 2, 5 and 8 respectively represent cb-Sgc8 prepared with Sgc8 with additional 9-base, 13-base or 17-base complementary sequences by slow chilling of denatured aptamers. Lanes 3, 6 and 9 respectively represent cb-Sgc8 prepared with Sgc8 with additional 9-base, 13-base or 17-base complementary sequences by fast chilling of denatured aptamers.

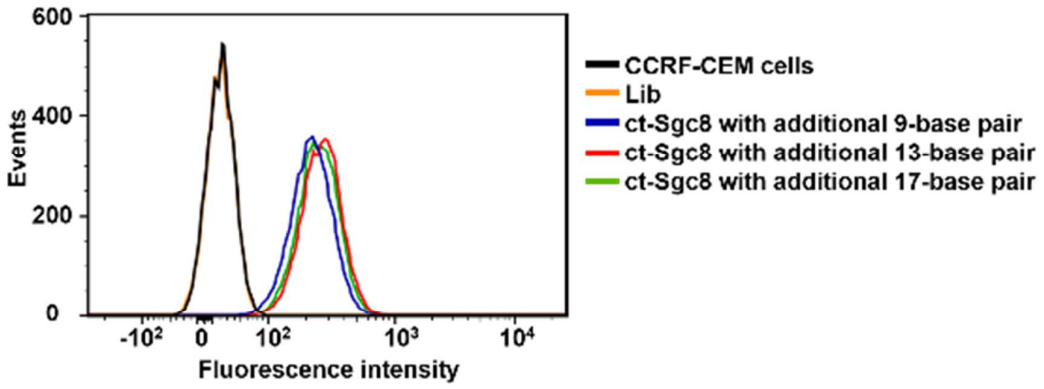

Figure S2. Flow cytometric assay of the binding ability of cb-Sgc8 with different additional base pairs on CCRF-CEM cells. The concentrations of FAM-labeled aptamers and library were 5 nM and 10 nM, respectively. The incubation temperature was 4 °C.

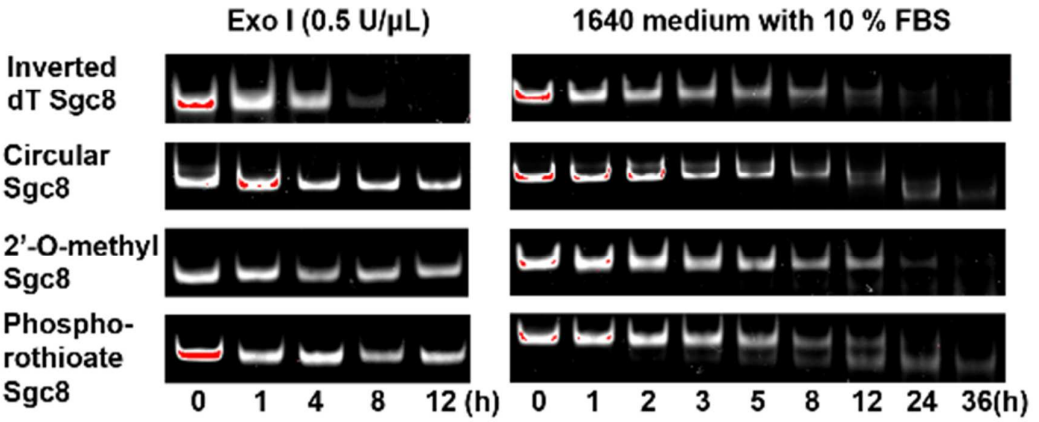

Figure S3. Stability analysis of circular aptamer and aptamers with different chemical modifications after treated with Exo I (left) and 10% FBS (right) for different times, as determined by PAGE. The concentrations of circular Sgc8 was 1 μM and the rest aptamers were 2 μM.

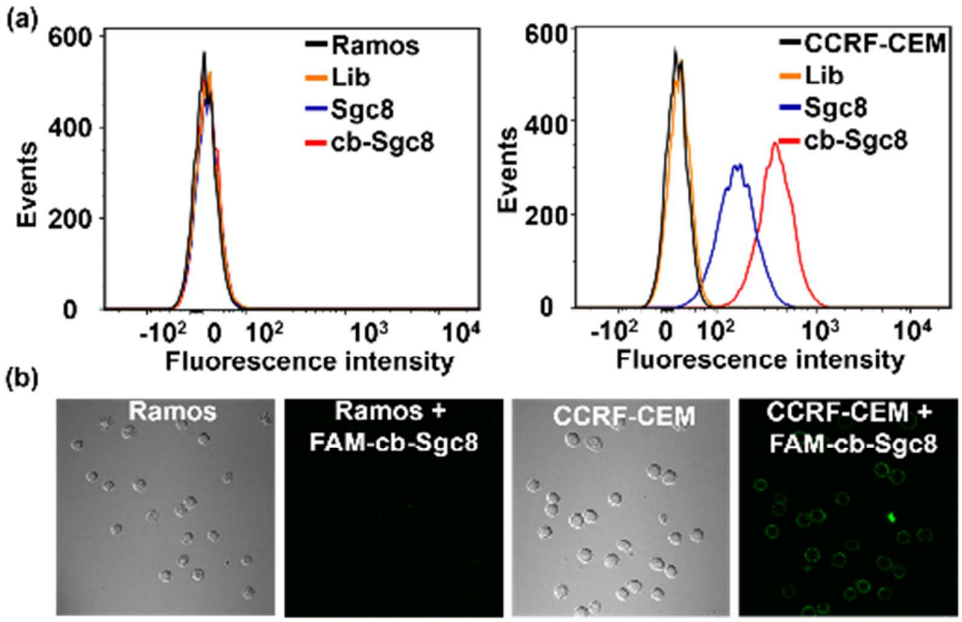

Figure S4. Specificity analysis of cb-Sgc8. Flow cytometry assay (a) and confocal microscopy assay (b) used for characterize the binding of FAM-labeled cb-Sgc8 on Ramos cells (left) and CCRF-CEM cells (right). The concentrations of Lib, Sgc8 and cb-Sgc8 were 300 nM, 300 nM and 150 nM, respectively. The incubation temperature was 4 °C.

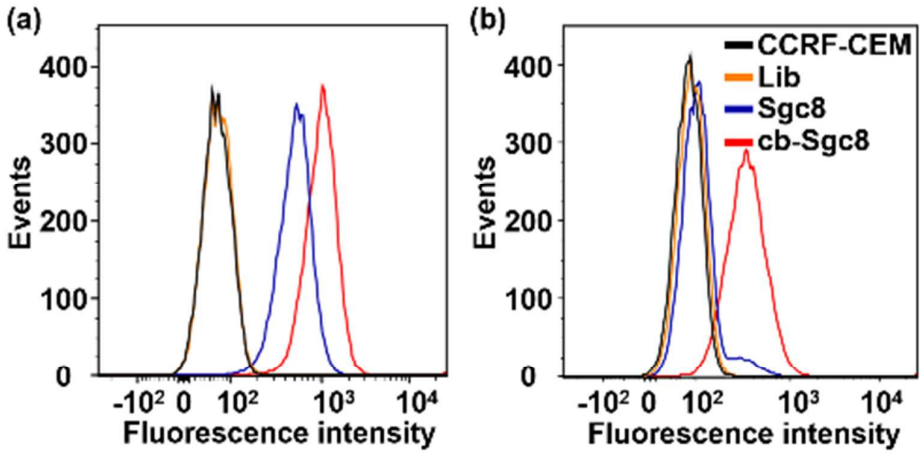

Figure S5. Flow cytometric assay of the binding ability (a) and internalization ability (b) of cb-Sgc8 in CCRF-CEM cells at 37 ℃. The concentrations of Lib, Sgc8 and cb-Sgc8 were 50 nM, 50 nM and 25 nM, respectively.

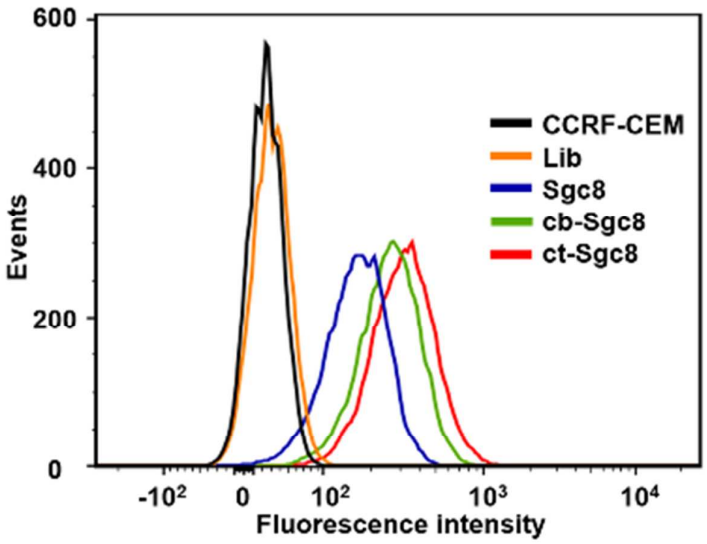

Figure S6. The binding ability of FAM-labeled Sgc8, cb-Sgc8 and ct-Sgc8 on CCRF-CEM cells with FAM-labeled library as control. The concentrations of aptamers and library were 10 nM and 5 nM, respectively. The incubation temperature was 4 ℃.

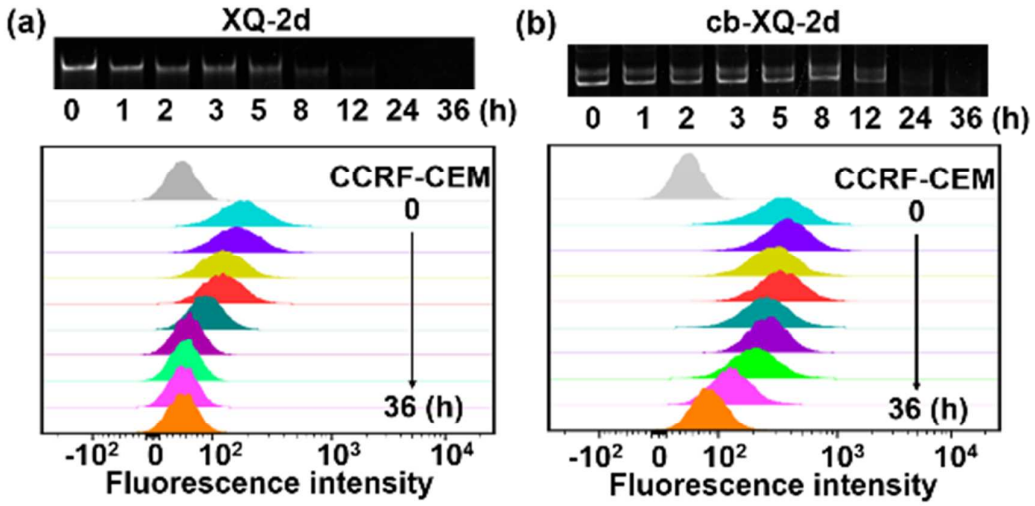

Figure S7. Stability analysis of XQ-2d (a) and cb-XQ-2d (b) after incubation in 10% FBS for different times, as determined by PAGE (upper) and flow cytometry assay (lower). The concentrations of XQ-2d and cb-XQ-2d in the PAGE analysis were 2 μM and 1 μM, respectively. The concentrations of XQ-2d and cb-XQ-2d in the flow cytometric assay were 100 nM and 50 nM, respectively. The incubation temperature was 4 °C.

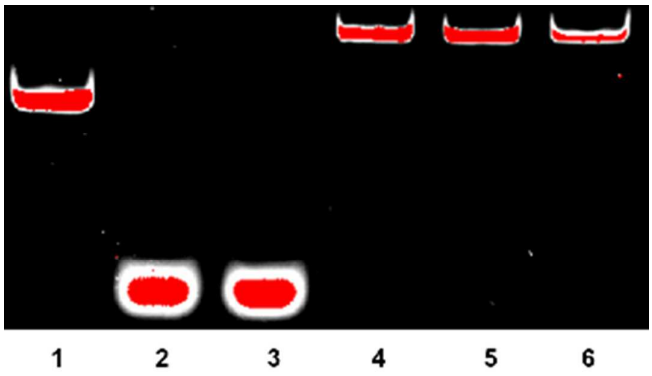

Figure S8. Stability analysis of cb-TD05 and TD05 after treatment with Exo I. The samples loaded in lanes 1, 2 and 3 were TD05, TD05 treated with 0.25 U/μL Exo I for 1 h, and TD05 treated with 0.5 U/μL Exo I for 1 h, respectively. The samples loaded in lanes 4, 5 and 6 were cb-TD05, cb-TD05 treated with 0.25 U/μL Exo I for 1 h, and cb-TD05 treated with 0.5 U/μL Exo I for 1 h, respectively. The incubation temperature was 37 °C. The concentrations of TD05 and cb-TD05 were 2 μM and 1 μM, respectively.

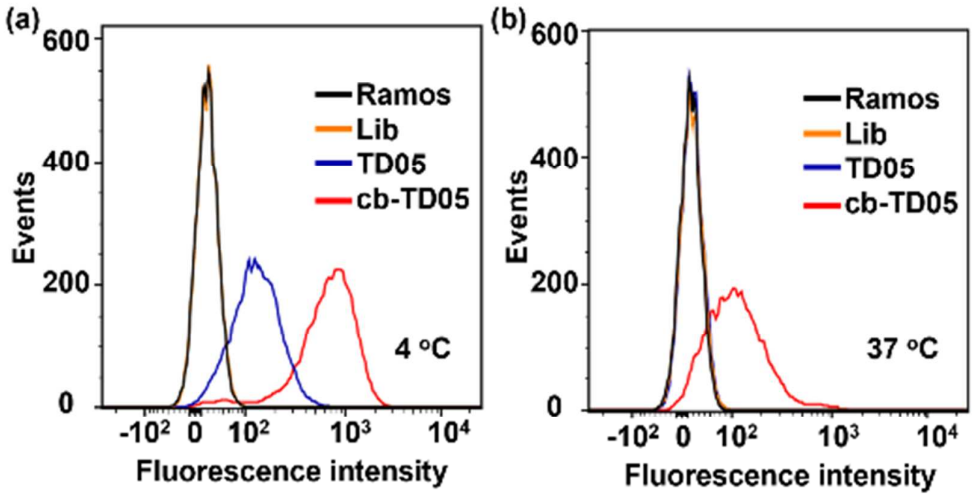

Figure S9. Flow cytometric assay of binding ability of cb-TD05 and TD05 on Ramos cells at 4 °C (a) and 37 °C (b). The concentrations of Lib, TD05 and cb-TD05 were 500 nM, 500 nM and 250 nM, respectively.

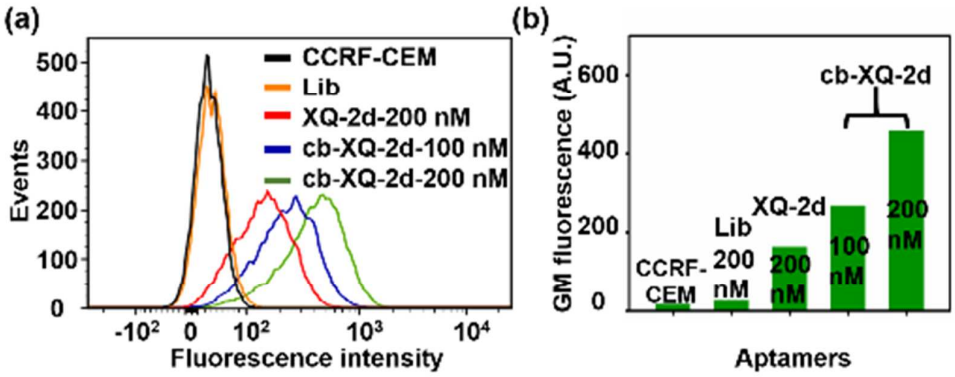

Figure S10. (a) Flow cytometric assay of binding ability of cb-XQ-2d and XQ-2d to CCRF-CEM cells (b) Summary of geometric mean fluorescence intensity of CCRF-CEM cells from (a).

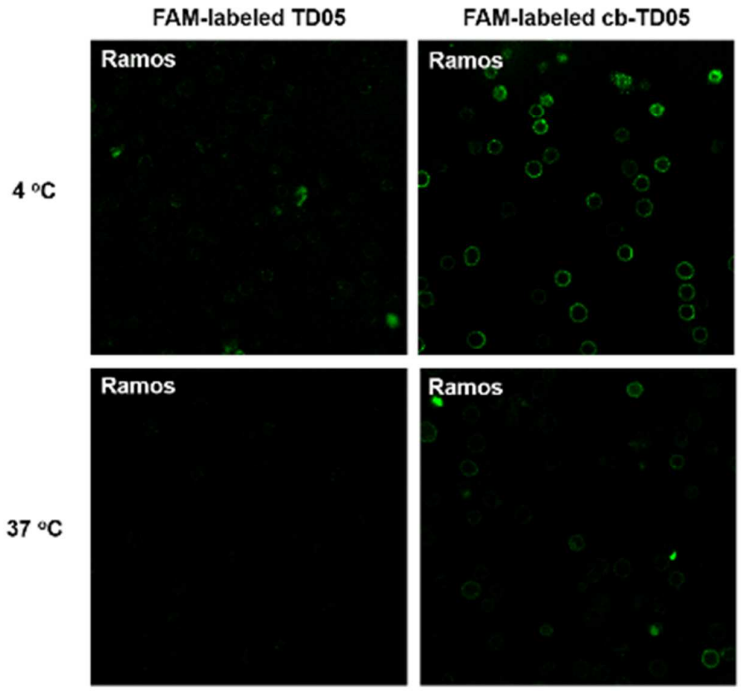

Figure S11. Confocal microscopy assay of binding ability of cb-TD05 and TD05 to Ramos cells at 4 °C (upper) and 37 °C (lower). The concentrations of library, TD05 and cb-TD05 were 500 nM, 500 nM and 250 nM, respectively.

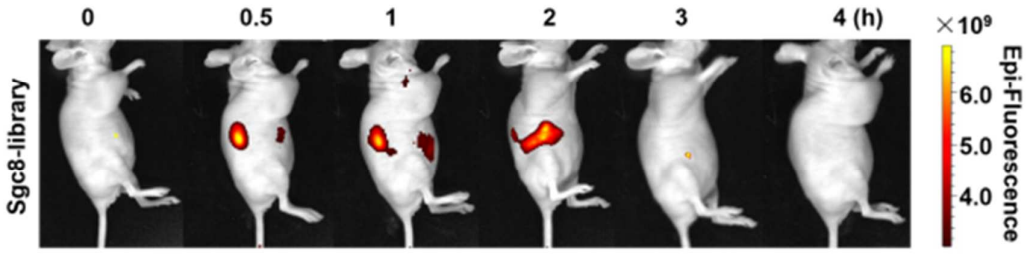

Figure S12. *In vivo* fluorescence imaging of CCRF-CEM tumor-bearing mice after Cy5-labeled library was injected through tail vein.