Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Circular Bivalent Aptamers Enhance the Activation of Regenerative Signaling Pathway for Repairing Liver Injury *In Vivo*

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EXPERIMENTAL SECTION

Reagents. All oligonucleotides were purchased from Sangon Biological Engineering Technology and Services & Co. Ltd. (Shanghai, China). Antibodies for ERK1/2(#4695), phosphor-ERK1/2 (Thr202/Tyr204, #4376), Actin (#4970), met (#8198), phosphor-met (#3077), Akt (#4691), phosphor-Akt (#4060) and Anti-rabbit IgG, HRP-linked Antibody (#7074) were supplied by Cell Signaling Technology. Recombinant human HGF was purchased by Novoprotein Scientific Inc. T4 DNA Ligase, Exonuclease Ⅰ and Exonuclease Ⅲ were purchased from Takara. Cy5 labeled recombinant human c-Met protein were purchased from Biotyscience Inc.

Construction of circular bivalent aptamer (CBA). The 5' phosphate-modified monovalent aptamers (MA-1 and MA-2) were used to construct circular bivalent aptamer (CBA). Briefly, the MA-1 and MA-2 were equivalently mixed in 1 x T4 DNA ligase buffer. The solutions were heated at 95 °C for 5 min, followed by chilling quickly to 16 °C to form bivalent aptamer (BA). Then, the BA was incubated with T4 DNA ligase at 16°C for 12 h to form initial CBA. The exonuclease (Exo Ⅰ and Exo Ⅲ) were added to remove unligased DNA. These enzymes were then denatured by heating at 90°C for 10 min. Finally, the CBA were extracted by PCR purification kit (transgenbiotech, EP101-01), according to manufacturer's instructions.

Urea polyacrylamide gel electrophoresis (Urea-PAGE). Urea denatured polyacrylamide gel electrophoresis was used to evaluate the construction of CBA. After treating a 1 μM sample (MA, BA, CBA, CBA with exonuclease, CBA with exonuclease and purification) with Loading Buffer, 12% Urea-PAGE was carried out in 1X TBE buffer at 80 V for 90 min. The DNA in gel was stained by gelred and imaged with ChemiDocTM Touch imaging system (Bio-Rad, America).

Stability assay. For analysis of the stability in exonuclease solution, MA, BA and CBA (0.2 μM, 20

μL) were incubated with 5 U exonuclease Ⅰ (Exo Ⅰ), 5 U exonuclease Ⅲ (Exo Ⅲ) for 70 min at 37°C. Then, each sample was analyzed using 3% agarose gels. The gels were run in 1x TBE at 80 V for 90 min and captured with ChemiDocTM Touch imaging system (Bio-Rad, America). For analysis of the stability in serum, MA, BA and CBA (1.5 μM, 20 μL) were incubated with RPMI-1640 with 10% FBS for 0, 1, 3, 5, 8, 24, 32, 48 h at 37°C. After incubation, samples were loaded onto 12% PAGE gel and run in 1X TBE buffer at 80 V for 90 min.

Binding affinity assay. The binding affinity was detected by MicroScale Thermophoresis (MST). Briefly, measurements were carried out with 200 nM Cy5-labeled Met protein in combination with CBA or BA at different concentrations (5000 nM, 2500 nM, 1250 nM, 625 nM, 313 nM, 156 nM, 78 nM, 39 nM, 20 nM, 10 nM, 4.8 nM, 2.4 nM, 1.2 nM, 0.6 nM, 0.3 nM, 0.15 nM), respectively. 10 μL DNA was mixed with 10 μL Met protein in buffer (PBS with the addition of 5 ‰ Tween 20) at 25°C for 20 min. All MST measurements were performed using Microscale Thermophoresis Monolith NT.115 (NanoTemper Technologies GmbH, Germany). Analysis of binding affinity was achieved via Kd fit formula using MO. Affifinity Analysis software.

Cell culture. Human normal hepatic L02 cells were cultured in RPMI-1640 medium (meilunbio, MA0215) with 10% FBS (Tocyto, UT81304) and 1% Penicillin-Streptomycin Solution (PS, Life Technologies). Human gastric cancer MKN-45 cells were cultured in Minimum Essential Medium (MEM, Corning) with 10% FBS and 1% PS. Cells were all cultured at 37°C in 5% CO_2.

Aptamer competition assays. MKN45 cells were seeded in confocal dishes at a density of 4×10^4/mL. Afterward, the cells were incubated with CBA or BA at room temperature. Cells were pretreated with 200 nM Cy5.5 labeled CBA or 200 nM FAM labeled BA for 15 min. After washed with PBS for three times, cells were imaged by Confocal laser scanning microscope (CLSM, Nikon

A1, Japan). Then, cells were incubated with 200 nM FAM labeled BA or 200 nM Cy5.5 labeled CBA for 15 min at room temperature. After washed with PBS for three times, cells were imaged again by CLSM.

Western blotting. For western boltting (WB) of cell lysate, L02 cells were seeded in 6-well plates (3×10^5 cells/well) overnight. Subsequently, cells were starved in RPMI-1640 medium supplemented with 0.5% BSA for 5 h. After starvation, cells were incubated with HGF or different oligonucleotide sample for 30 min at 25°C. After the incubation, cells were lysed and harvested total proteins using Cell lysis buffer for Western and IP (Beyotime, P0013) supplemented with Protease Inhibitor Cocktail (MCE, HY-K0011) and Phosphatase Inhibitor Cocktail (Roche,4906845001). The protein concentration was measured by the BCA assay. Then, the protein samples were separated by 5%-8% SDS−PAGE gels and transferred onto a PVDF membrane. The membrane was reacted with 5% milk/TBST for 2 h to shield nonspecific binding sites. Afterwards, the membranes were reacted with primary antibodies overnight at 4°C and secondary antibody for 1.5 h at room temperature, respectively. After that, the membranes were probed using BeyoECL Moon (Beyotime, P0018FS). Finally, the chemiluminescent images were captured using ChemiDocTM Touch imaging system (Bio-Rad, America).

Cell proliferation assay. L02 cells were seeded in 96-well plates (2×10^3 cells/well) and cultured overnight. After serum-starved for 5 h, cells were incubated with CBA at various concentration (1 nM，2.5 nM，5 nM，8 nM，10 nM，30 nM, 50 nM). After 2 days, cell proliferation was evaluated by cell counting kit-8 (CCK-8) according to the manufacturer's instructions.

Animals. The female BALB/c mice were purchased from GemPharmatech. All animal procedures were implemented in compliance with the guidelines of the Institutional Animal Care and Use

Committee of Minjiang University (Approval ID: 20210601R).

Biodistribution assay. To study the biodistribution of CBA and BA, BALB/c mice (average weight of 20 g) were injected with 0.5 nmol Cy5.5-labeled CBA and BA for varying time periods (15 min, 2 h, 8 h, 24 h, 48 h). After appropriate time, the organs (heart, liver, spleen, lung and kidney) were excised and imaged using Ami X optical imaging system (Spectral Instruments, America) (excitation/emission = 676/705 nm).

Analysis of Met activation in liver. The HGF(2 μg) and CBA, BA (0.5 nmol) were injected via the tail vein to BALB/c mice. After 15 min, the liver samples were excised for WB and Immunohistochemistry (IHC). For WB, the liver samples were homogenized to harvest proteins by using disposable homogenizer with a lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein concentration was measured by the BCA assay, separated by 5%-8% SDS−PAGE gels and transferred onto a PVDF membrane. The membrane was reacted with 5% milk/TBST for 2 h, following primary antibodies met, phosphor-met and actin overnight at 4°C and secondary antibody for 1.5 h at room temperature, respectively. Finally, the chemiluminescent images were captured using ChemiDocTM Touch imaging system (Bio-Rad, America). For IHC, the livers were collected, fixed in 4% PFA and dehydrated in sucrose. After that, the liver tissues were embedded in OCT, cut into sections (8 μm thickness) using CryoStar NX50 OPD (ThermoFisher Scientific, America) and stained with phosphor-Met. The obtained tissue sections were imaged by using a microscope (Nikon, Japan).

Treatment of acute liver injury mouse models. To construct the acute liver injury mouse models, the female BALB/c mice were injected intraperitoneal with acetaminophen (APAP) 450 mg/kg following a 18 h fasting according to previous report.[1] After 12 h of APAP treatment, the CBA or

BA (2.5 nmol) was injected once every 12 h, twice in total via the tail vein to mice. After 12 h of second injection, the blood samples and liver tissues were harvested for analysis. The serum alanine aminotransferase (ALT) and aspartate amino transferase (AST) levels were quantified by using the ALT and AST detection kits (Nanjing Jiancheng Biological Engineering Institute), following the manufacturer's instructions. The harvested liver tissues were divided into partitions for hematoxylin and eosin (H&E) histopathological analysis, and for TUNEL assay according to the previous reports.[2]

Statistical analysis. The results were presented as mean value with a standard deviation (± SD) of at least 3 independent experiments in each group. Statistical analysis was performed using student's t test for comparison differences between various treatment groups. The p-value<0.05 was assumed as statistically significant. *P < 0.05, **P < 0.01, n.s. = not significant.

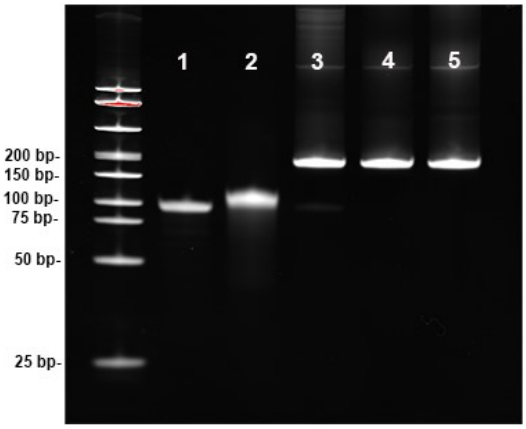

Figure S1. Construction of CBA before and after exonuclease treatment and purification, as determined by Urea denatured PAGE gel electrophoresis (lane 1: MA, lane 2: BA, lane 3: CBA without purification, lane 4: CBA treated with exonuclease, lane 5: CBA treated with exonuclease and purification).

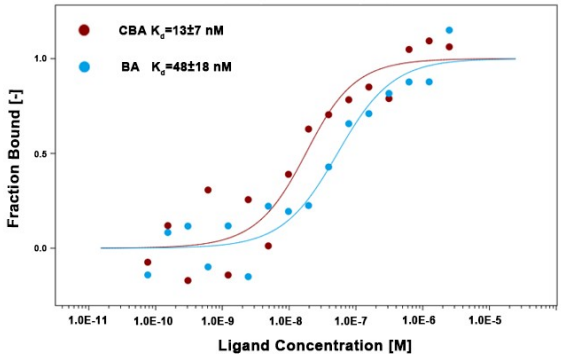

Figure S2. Met-binding affinity analysis of CBA and BA by MST.

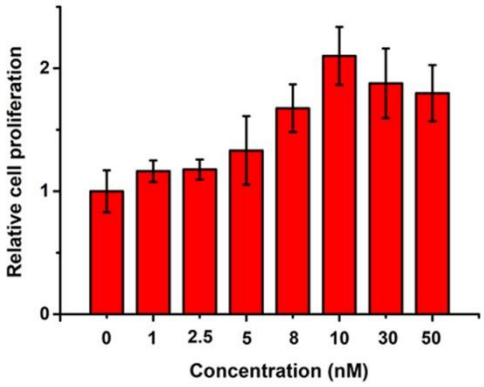

Figure S3. The proliferation of L02 cells stimulated by CBA, measured by Cell Counting Kit-8 (n=3).

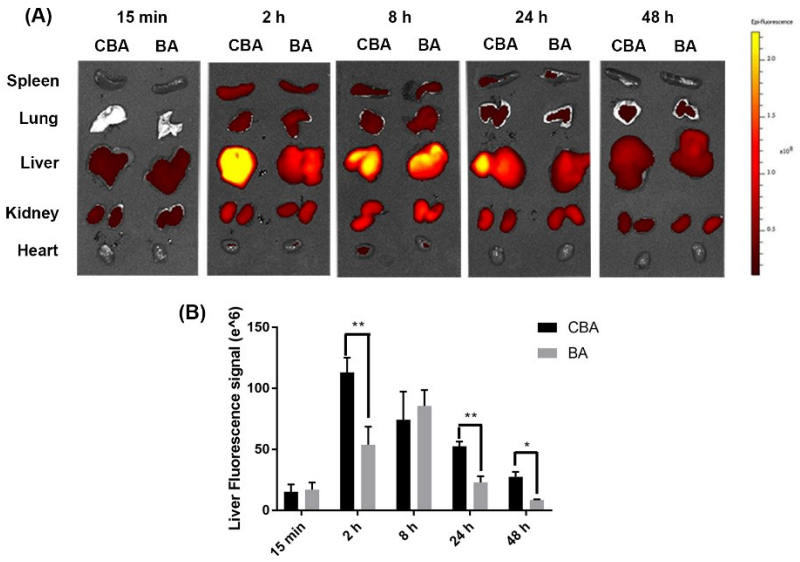

Figure S4. (A) Representative fluorescence image of the organs after tail vein injected with Cy5.5–labeled CBA and BA (0.5 nmol) for different time periods. (B) Quantitative analysis of the liver

fluorescence signals for different time periods(n=3). Significance (**$P < 0.01$; *$P < 0.05$).

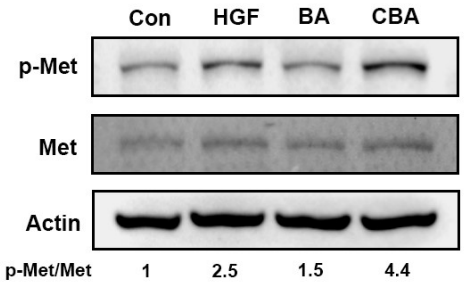

Figure S5. WB analysis of Met phosphorylation in the mice liver after intravenous injection of HGF (2 μg), BA (0.5 nmol) and CBA (0.5 nmol), respectively.

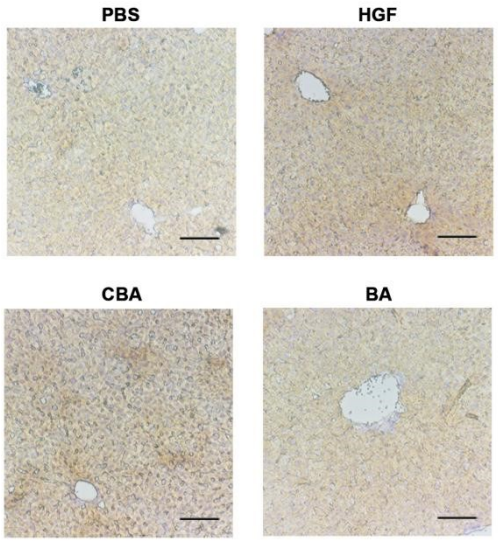

Figure S6. Immunohistochemistry of p-Met in the mice liver after intravenous injection of HGF (2 μg), BA (0.5 nmol) and CBA (0.5 nmol), respectively. Scale bars: 100 μm.

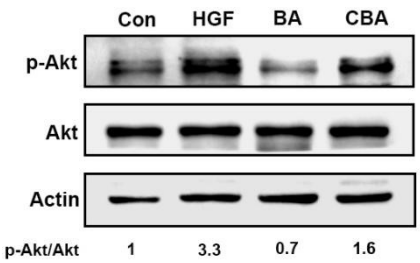

Figure S7. WB analysis of Akt phosphorylation in the mice liver after intravenous injection of HGF (2 μg), BA (0.5 nmol) and CBA (0.5 nmol), respectively.

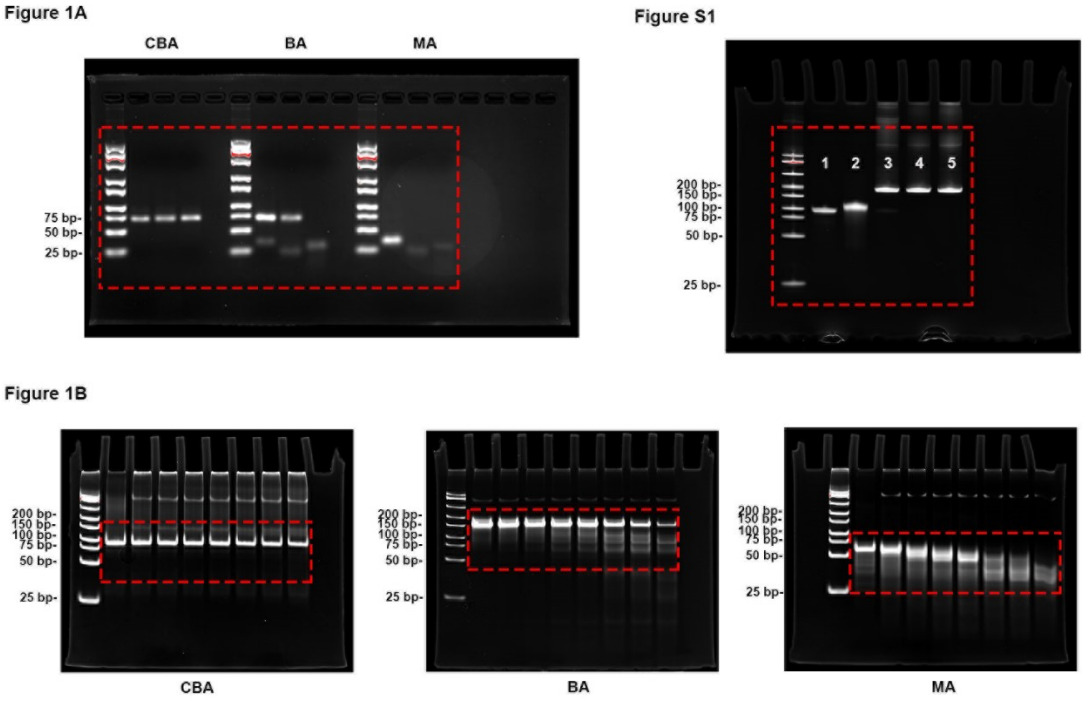

Figure S8. Raw images of agarose (Figure 1A), PAGE (Figure 1B) and Urea denatured PAGE gel (Figure 1S) electrophoresis.

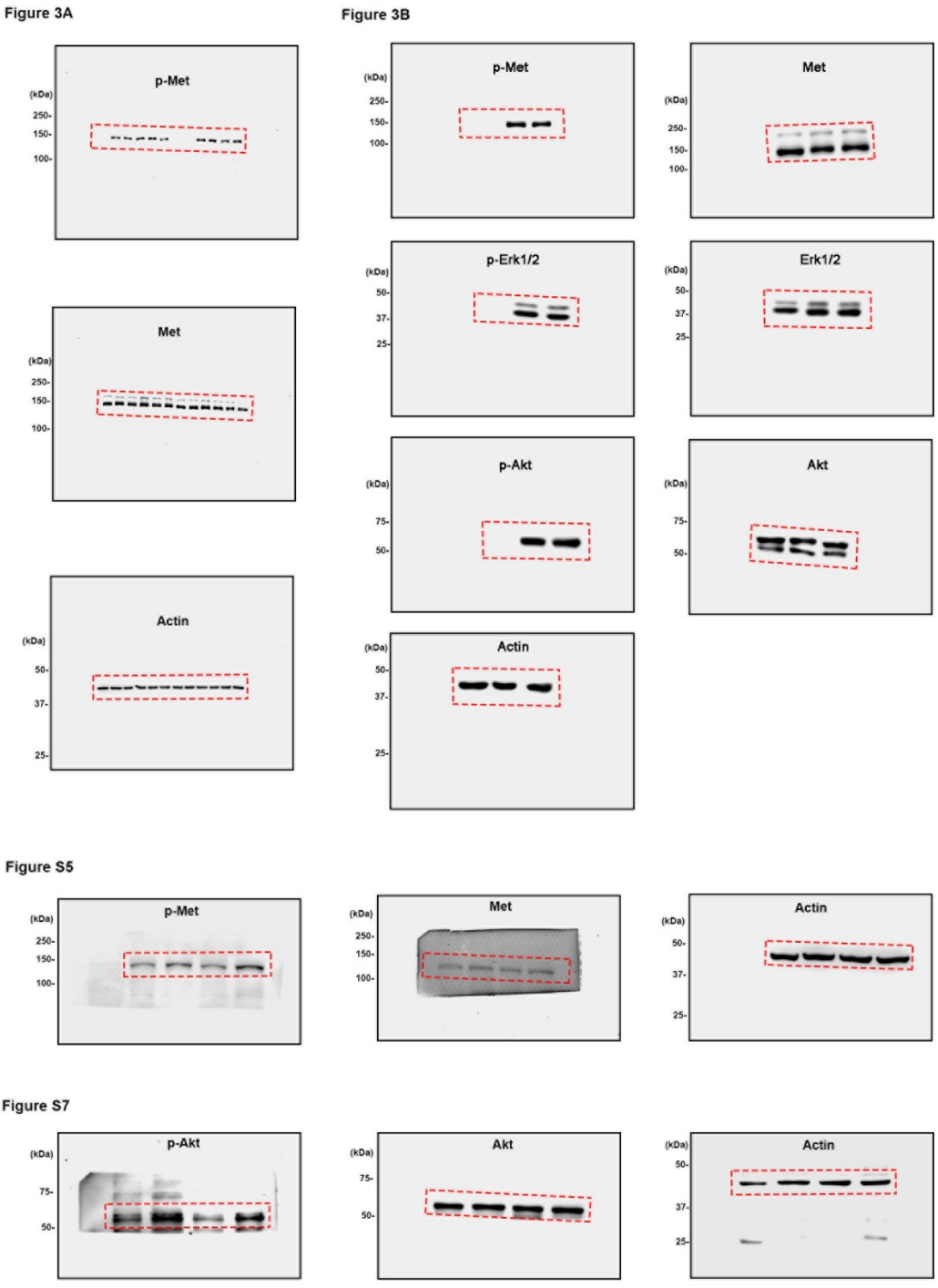

Figure S9. Raw images of Western blotting shown in Figure 3, S5, and S7.

Table S1. The sequences of DNA employed in this work.[a]

Oligo Name	Sequence (5' to 3')
MA-1	Phosphate-ATC AGG CTG GAT GGT AGC TCG GTC GGG GTG GGT GGG TTG GCA AGT CTG ATC TGT AGA ACG TTA TCA TA
MA-2	Phosphate-ATC AGG CTG GAT GGT AGC TCG GTC GGG GTG GGT GGG TTG GCA AGT CTG ATT ATG ATA ACG TTC TAC AG

a The sequence of Met-binding aptamer were colored red. The complementary sequences were colored black.

REFERENCES

1 a) C. M. Li, Y. N. Ming, W. T. Hong, Y. Y. Tang, X. H. Lei, X. B. Li and Y. M. Mao, *Toxicol. Lett.* 2018, **283**, 69-76; b) H. Qian, Q. Y. Bai, X. Yang, J. Y. Akakpo, L. L. Ji, L. Yang, T. Rulicke, K. Zatloukal, H. Jaeschke, H. M. Ni and W. X. Ding, *Acta Pharm. Sin. B* 2021, **11**, 3791-3805.

2 F. Rizvi, E. Everton, A. R. Smith, H. Liu, E. Osota, M. Beattie, Y. Tam, N. Pardi, D. Weissman and V. Gouon-Evans, *Nat. Commun.* 2021, **12**, 613.