Electronic Supporting Information (ESI) for

Detection of interaction between an RNA aptamer and its target compound in living human cell using 2D in-cell NMR

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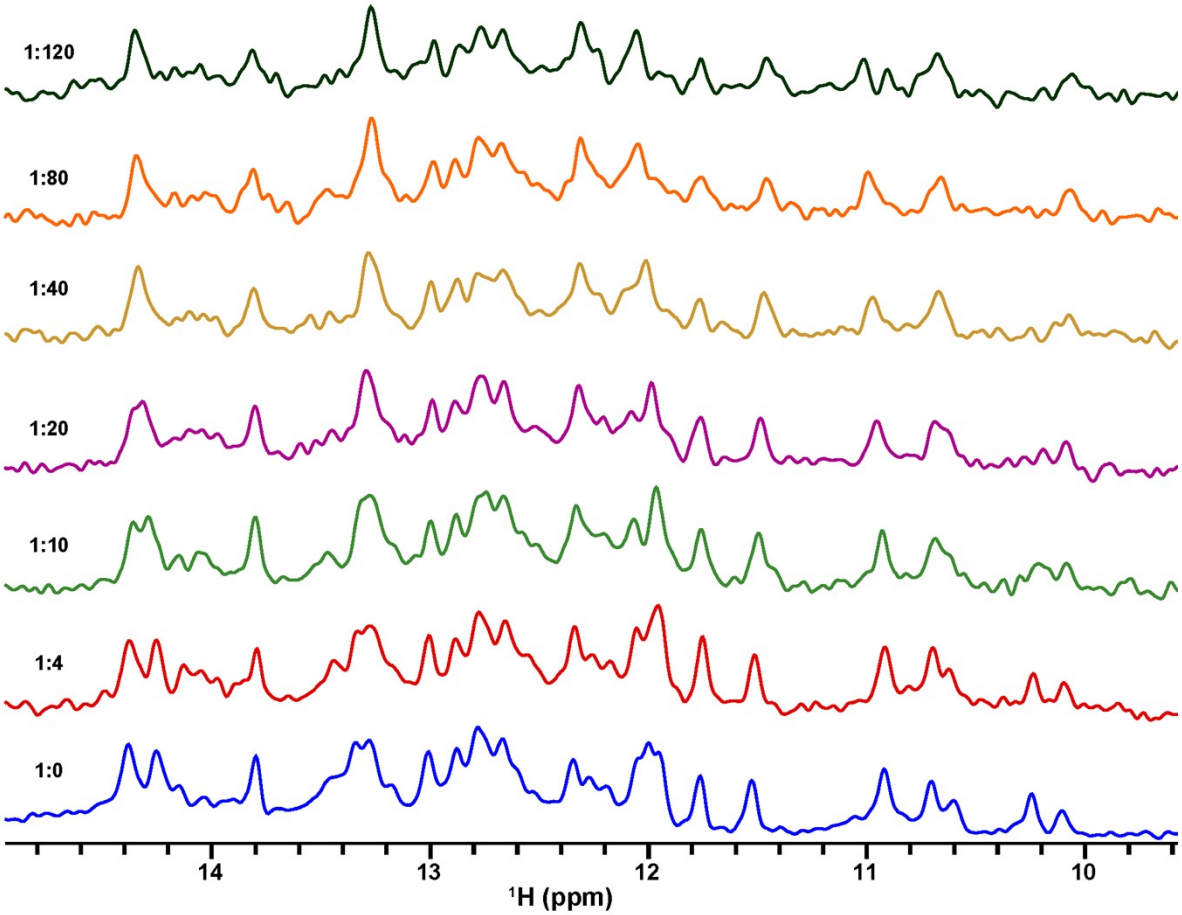

Fig. S1 ^{1}H-NMR spectra of the RNA aptamer for HIV-1 Tat titrated with argininamide. Imino regions of ^{1}H-NMR spectra of the RNA aptamer (20 μM) titrated with argininamide in TB at 10°C. Molar ratios of [TA_36]:[argininamide] = 1:0, 1:4, 1:10, 1:20, 1:40, 1:80, and 1:120 are indicated.

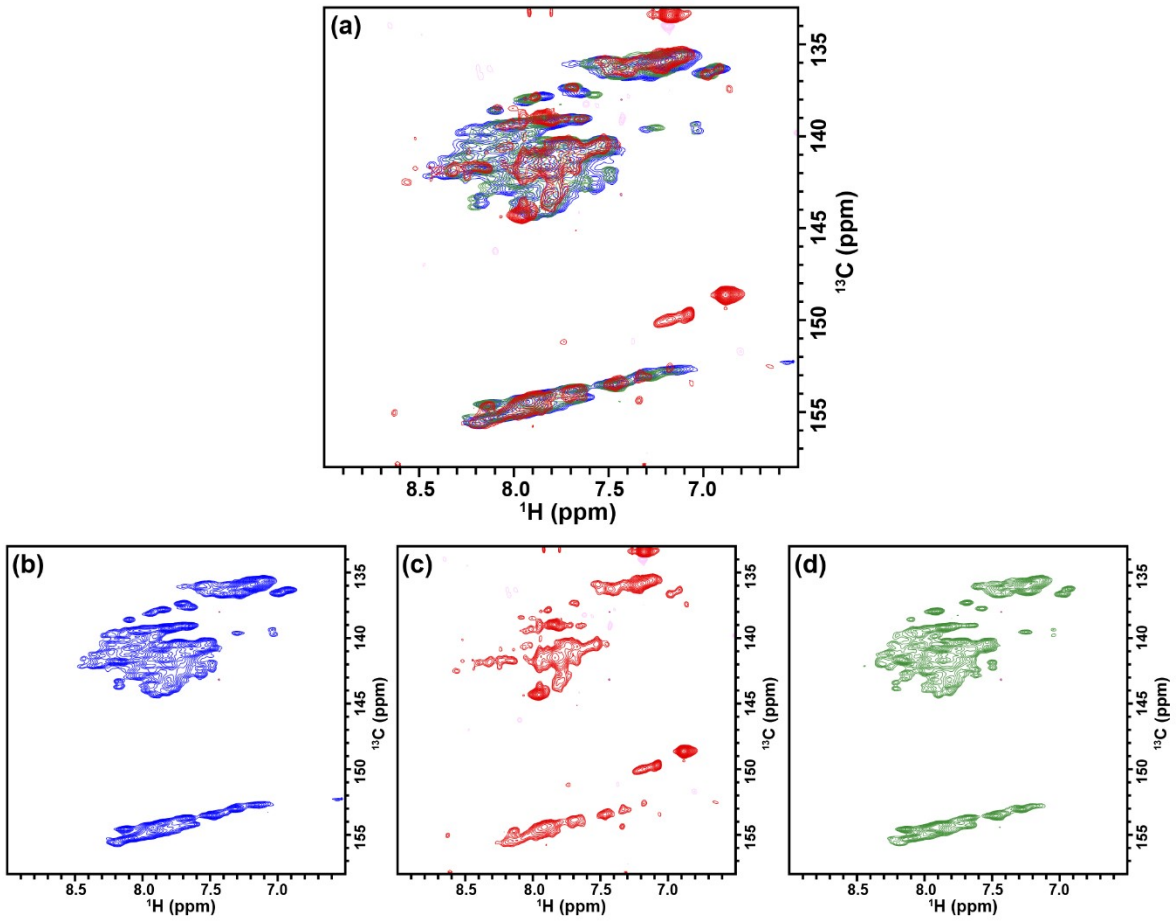

Fig. S2 2D ^{1}H-^{13}C HMQC spectra of TA_36 by itself and in a complex with argininamide recorded in in vitro and in-cell conditions. The C8-H8/C6-H6/C2-H2 region is shown. Two in vitro and one in-cell 2D ^{1}H-^{13}C HMQC spectra are superimposed (a). The in vitro 2D ^{1}H-^{13}C HMQC spectra were recorded for TA_36 dissolved in TB by itself (blue) and with argninamide (green). An in-cell 2D ^{1}H-^{13}C HMQC spectrum was recorded for TA_36, which was introduced into HeLa cells with argininamide (red). (b-d) Each of the spectra superimposed in (a) is presented individually; the same colors are used as in (a).

■ Materials and Methods

Construction of an expression vector

The DNA sequence encoding TA_36 with a scaffold was purchased from Integrated DNA Technologies, Inc. (Iowa, USA). The DNA sequence contains those for TA_36, tRNA, a Sephadex aptamer (Fig. 1a, grey), two hammerhead (HH) ribozymes, and two variable arms (5'CCCUCGAA and CGAGGGA-3'), which are schematically shown in Fig. 1. The DNA sequences for tRNA, the Sephadex aptamer, and two HH ribozymes were taken from the ref. [1] The DNA sequence encoding TA_36 with the scaffold was subcloned into pETUK (BioDynamics Laboratory. Inc., Tokyo, Japan) between the Xbal and BamHI restriction sites.

Preparation of isotopically labeled RNA

Escherichia coli, BL21-Gold (DE3) cells were transformed with the constructed plasmid and grown in 1 L of M9 minimal medium containing 1 g/L ^{15}N-NH_4Cl and 1 g/L ^{13}C-glucose as the sole nitrogen and carbon sources, respectively, to OD_{600} of 0.6. The expression of TA_36 with the scaffold was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C, and the cultures were harvested 6 h after induction by centrifugation (30,000 g) for 20 min at 4°C. To extract the RNAs, 15 mL acidic phenol (saturated in 10 mM Tris-HCl) was added to the collected cells, which were then agitated gently for 10 min at 4°C. After centrifugation, the aqueous phase was collected, to which 1.5 mL 3 M sodium acetate (pH 5.2) and 49.5 mL ethanol were added and mixed in vigorously. After storage at -20°C for 1 h, the solution was centrifuged. The precipitate containing RNA was air-dried and then dissolved in a solution comprising 50 mM Tris-HCl (pH 8.1) and 100 mM NaCl. This RNA solution was heated to 95°C for 10 min and then gradually cooled to room temperature in 1.5 h. Next, to cleave and isolate the TA_36 from the scaffold portion, 5 mM $MgCl_2$ was added, by which the HH ribozyme portion was activated, followed by incubation at 37°C for 1 h. A final concentration of 8M urea was added to this RNA solution, which was then loaded onto tandemly connected three 5 mL HiTrap Q HP columns (Cytiva, Massachusetts, USA) at the flow rate of 1 mL/min. The bound fraction was eluted with a linear gradient of 0.1 – 1.0 M NaCl. All the collected fractions were analysed by denaturing urea polyacrylamide gel electrophoresis (8 M urea in 20% polyacrylamide gel). The gel was stained with SYBR Gold. The fractions containing isolated TA_36 were pooled and dialyzed against ultrapure water. The dialysate was then lyophilized and stored.

In vitro NMR measurements

The TA_36 was dissolved to a final concentration of 50 µM in Transfer Buffer (TB: 25 mM HEPES-KOH (pH 7.4), 115 mM CH_3COOK, 2.5 mM $MgCl_2$), which was then supplemented with 5% D_2O and 10 µM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). This sample solution was heated at 95°C for 10 min and then cooled to 4°C at a rate of -1°C/min using a TaKaRa RCR Thermal cycle Dice Gradient (Takara, Kusatsu, Japan).

1D ^{1}H NMR spectra were recorded by the band-selective optimized-flip-angle short-transient (SOFAST)[2] technique with the band-selective excitation PC9 and refocusing REBURP pulses centered at the imino proton region. The acquisition time was 1.5 min. 2D ^{1}H-^{15}N SOFAST-HMQC spectra were recorded with 16 scans, 200 msec interscan delays, and 1376 (t2) × 256 (t1) complex points. 2D ^{1}H-^{13}C SOFAST-HMQC spectra were recorded with 256 scans, 200 ms interscan delays, and 1024 (t2) × 256 (t1) complex points. The acquisition times were 20 min for 2D ^{1}H-^{15}N SOFAST-HMQC, and 290 min for 2D ^{1}H-^{13}C SOFAST-HMQC. All NMR spectra were recorded at 10°C using a Bruker BioSpin AVANCE III HD 600 spectrometer equipped with a cryogenic probe.

In-cell NMR measurements using a bioreactor system

For the sample preparation in in-cell NMR experiments, the RNA and ligand were introduced into HeLa cells by the electroporation method.[3,4,5] The 2D ^{13}C, ^{15}N-labeled TA_36 was dissolved in TB, followed by heating at 95°C for 10 min and then cooling to 4°C at a rate of -1°C/min. Then, the TA_36 solution was mixed with non-labeled argininamide. The final concentrations of TA_36 and argininamide were 1 mM and 4 mM, respectively. HeLa cells were harvested and washed twice with phosphate-buffered saline (PBS). The HeLa cells were centrifuged and the cell pellet was suspended in the TA_36 solution. The suspension was divided into three electroporation cuvettes (2 mm gap), each cuvette containing 150 µL of the suspension with 1.5 × 10^7 cells. The suspension in the cuvettes was incubated on ice for 10 min prior to electroporation. The electroporation was conducted with an NEPA21 Super Electroporator system (Nepa Gene Co., Ltd., Chiba, Japan) using two poring pulses (125 V) with a 50 msec interval and then five transferring pulses (20 V) with a 50 msec interval. After electroporation, the cells were transferred immediately to pre-warmed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and then incubated at 37°C for 15 min for recovery of cells.

In-cell NMR experiments were carried out with a bioreactor system[6,7] as described previously.[8] The electroporated cells were washed with 0.9 × Leibovitz's L-15 medium, and a 80% slurry of the electroporated cells was mixed with the same volume of 0.9 × Leibovitz's L-15 medium containing 3% of low-melting Seaprep agarose (Lonza, Basel, Switzerland), 10% D_2O, and 10 μM DSS to finally yield a mixture of 40% cells and 1.5% agarose. The mixture was transferred *via* a syringe to a polytetrafluoroethylene (PTFE) tube of 0.5 mm inner diameter and then incubated on ice for 10 min. The solidified agarose gel filament containing cells in the PTFE tube was extruded into a 5 mm NMR tube with 140 μL of 3% agarose gel containing 0.9 × L-15 medium, 10% D_2O, and 20 μM DSS at its bottom as the lower gel. In addition, in a silicon tube of 1 mm inner diameter, another 140 μL of 1.5% solidified agarose gel filament without cells was extruded as the upper gel and finally, the NMR tube was manually centrifuged.

During the in-cell NMR measurements, 0.9 × Leibovitz's L-15 medium containing 10% D_2O and 10 μM DSS was supplied from a syringe pump at a flow rate of 50 μL/min through a PTFE tube that was connected to the NMR tube (inlet PTFE tube). Fresh 0.9 × Leibovitz's L-15 medium was delivered into the NMR tube to maintain a healthy environment for HeLa cells, with draining of the exhausted medium into a waste bottle through Inlet and outlet PTEF tubing.

1D ^{1}H NMR spectra were recorded by the SOFAST[2] technique with PC9 and REBURP pulses centered at the imino proton region. The acquisition time was 40 min. 2D ^{1}H-^{15}N SOFAST-HMQC spectra were recorded with 16 scans, 200 msec interscan delays, and 1376 (t2) × 64 (t1) complex points. ^{1}H-^{13}C SOFAST-HMQC spectra were recorded with 256 scans, 200 ms interscan delays, and 1024 (t2) × 128 (t1) complex points. The acquisition times were 100 min for 2D ^{1}H-^{15}N SOFAST-HMQC, and 145 min for 2D ^{1}H-^{13}C SOFAST-HMQC. All NMR spectra were recorded at 10°C using a Bruker BioSpin AVANCE III HD 600 spectrometer equipped with a cryogenic probe.

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