Electronic supplementary information(ESI)

Inhibition of homophilic dimerization and disruption of cell adhesion by P-cadherin-specific small molecules from SPR-based assays

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1. Material and Method

1.1 Compounds

Fragment library (Maybridge Ro3 fragment library) was purchased from Maybridge. Each fragment was dissolved in DMSO and stored at −30 °C. Before the assay, the compounds were transferred from the mother plate to the plate used in the assay.

1.2 Expression and purification of classical cadherin constructs and TSP7

All of the classical cadherin constructs (P-cadherin EC12, P-cadherin REC12, N-cadherin REC12, E-cadherin REC12) were expressed in *Escherichia coli* Rosetta2 (DE3) cells (Merck Millipore). *E. coli* was transformed with expression vector, inoculated in 6 mL of LB medium containing 50 mg/mL kanamycin and 34 mg/mL chloramphenicol and pre-cultured at 37 °C for 16 hours. Cells are transferred to 1 L of the fresh LB medium containing the antibiotics stated above and cultured at 37 °C for about 4 hours to get the $O.D._{600}$ value 0.4~0.6. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C for around 16 hours. Cells were collected by centrifugation at 7000 g, for 10 min at 4 °C. After suspending the *E. coli* pellet with a binding buffer (20 mM Tris-HCl, 300 mM NaCl, 3 mM $CaCl_2$, 20 mM Imidazole pH 8.0), cells were sonicated. The lysate was centrifuged at 40000 g, for 30 min at 4 °C. The

supernatant was loaded to the Ni-NTA column, which was equilibrated with the binding buffer beforehand. Each classical cadherin construct was eluted with the elution buffer (20 mM Tris-HCl, 300 mM NaCl, 3 mM $CaCl_2$, 300 mM Imidazole pH 8.0). The elution protein solution was further purified with size exclusion chromatography (SEC). The elution sample was loaded to a Hiload26/60 Supderdex-200 column which was equilibrated with SEC buffer (10 mM HEPES-NaOH, 150 mM NaCl, 3 mM $CaCl_2$ pH 7.5).

In general, TSP7 was purified using the same method as for the classical cadherin constructs. *E. coli* BL21 (DE3) was used and the binding buffer contained 20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole pH 8.0. The elution buffer was 20 mM Tris-HCl, 500 mM NaCl, 100 mM Imidazole pH 8.0. In SEC process, a Hiload 26/60 Supderdex-75 column was used. SEC buffer was 50 mM Tris-HCl, 200 mM NaCl pH 8.0.

1.3 Direct binding assay to REC12 constructs in SPR

The direct binding assay was carried out for a first screen and confirmation of specificity to P-cadherin. All the experiments were performed with Biacore 8K instrument (GE Healthcare). Biotinylated monomeric construct of each classical cadherin was immobilized on Sensor Chip SA, to get approximately 3000 RU. Each compound was

diluted in running buffer (10 mM HEPES-NaOH, 150 mM NaCl, 3 mM CaCl2, 0.05% Tween 20, 5% DMSO) to a final concentration of 500 μM. Association time was 20 seconds, and dissociation time was 20 seconds. 1 M Arg-HCl pH 4.4 was used to regenerate the ligand. Negative (buffer alone) and positive control (TSP7) experiment was performed periodically to check the stability of the ligand. The binding level of each compound was normalized against buffer and TSP7 responses. A dose-response experiment was performed to confirm the specificity to P-cadherin. Each hit compound was diluted into a 2-folds dilution series (500 μM to 31.3 μM). Kinetic parameters were evaluated in Biacore 8K evaluation software.

1.4 ABA assay in SPR

ABA assay was carried out as a part of the second screening experiments to select compounds which inhibit the homodimerization. ABA injection is a feature in Biacore 8K (GE Healthcare), making it possible to inject two different solutions in a sequence of three segments. In previous experiments we found that EC12 bound non-specifically to the Sensor Chip CM5. To prevent this, we selected Sensor Chip C1 for this assay. EC12 was immobilized via amine coupling reaction and resulted in an immobilization level of approximately 120 RU. Solution A; 1 μM EC12, and solution B; 500 μM of each

compound. Solution A and B were diluted in running buffer (the same as that of 3.3). A contact time of 180 seconds was used for solution A, and 120 seconds for solution B. The longer contact time for solution A in comparison to solution B was made to allow the surface to form as much EC12 dimers as possible. The decrease in binding level of B part was evaluated by visual inspection of the sensorgrams.

1.5 Monomer detecting assay in SPR

Monomer detecting assay was performed to select compounds which can shift equilibrium to the monomer part. The experiment was also performed in Biacore 8K. His_6-tag TSP7 was captured to a level of approximately 2000 RU on Sensor Chip Ni-NTA in each cycle. As analyte, a mixture of 1.6 μM EC12 and each compound was used. Contact time was 120 seconds, and dissociation time was 600 seconds. Negative and positive control experiments were performed in the beginning and end of the assay. Binding data was evaluated in the Biacore 8K evaluation software. Binding levels were normalized by using the positive and negative controls included in the assay.

1.6 Cell aggregation assay

Inhibitory activity of hit compounds was investigated by cell aggregation assay. CHO

cells expressing GFP fusion P-cadherin was used. After Trypsin calcium (TC) treatment and the wash with HCMF buffer, 5.0 $\times 10^4$ cells were seeded to 24 well plate. Cell aggregation reaction was initiated by adding the Ca^{2+} to the cells at 37 °C in a rotor spun at 80 rpm for 30 min. As control experiments, 1 mM EDTA or 1 μM TSP7 was added to the well. Compounds were diluted in HCMF buffer containing 2% DMSO to a final concentration of 1 mM. After the reaction, images were taken using an Evos XL core with a 4×0.13 NA objective lens (Thermo Fisher).

1.7 Cell adhesion assay

Inhibitory activity of hit compounds for living cells was analyzed in cell adhesion assay. CHO cells expressing GFP fusion P-cadherin were used here too and their pictures were taken with an In Cell Analyzer 2000 (GE Healthcare). 2.0×10^4 Cells were seeded to 96 well plate (Greiner) and incubated for 2 days, at 37 °C, 5% CO_2 before the assay. Just before the assay, medium (F-12 medium containing 10% FBS, 1% L-Glu, 1% penicillin-streptomycin) in each well is replaced with the new one containing each concentration of a compound. After the compounds were added, cells were incubated for another half an hour under the same condition. Cell nuclei were immunostained by Hoechst 33342 (Thermo Fisher). Pictures were taken with the In Cell Analyzer 2000 using a 60 ×0.7 NA

objective lens, and analyzed using Developer Toolbox. To detect the GFP fusion P-cadherin on the interface of cell adhesion, object segmentation was set up (kernel size: 5, sensitivity: 60). Also, for the detection of nuclei, nuclear segmentation was set up (minimum target area: 511, sensitivity: 50).

2. Supplementary figures

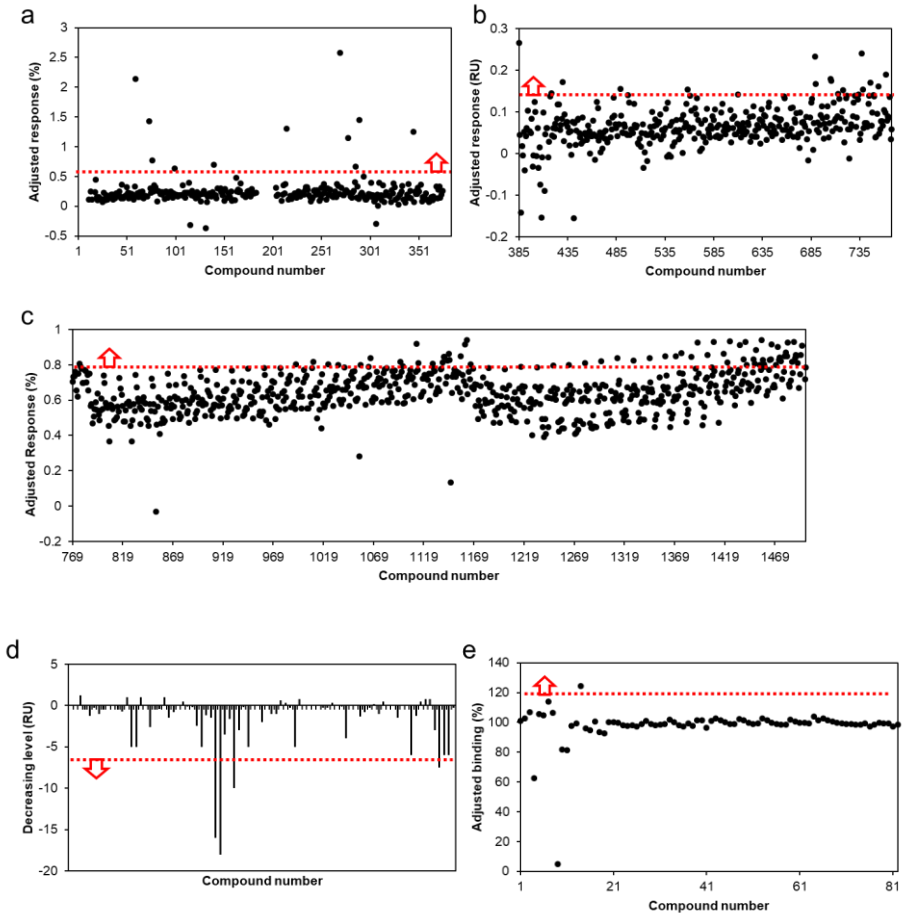

Fig. S1 Fragment screening by SPR. A set of red line and arrow indicates a hit region. (a)~(c) Adjusted binding level of 1500 fragment compounds to REC12 immobilized on Sensor Chip SA. 100 % means the binding level of TSP7, although not shown in the figure above. Total four 384 well plates were screened, and (a), (b), and (c) shows the result of plate No.1, No. 2, and No. 3, 4 respectively. (d) Decreasing response at B part from ABA assay for all the hits from the first screening. (e) Adjusted binding to the TSP7 from the monomer detecting assay for all the hits from the first screening.

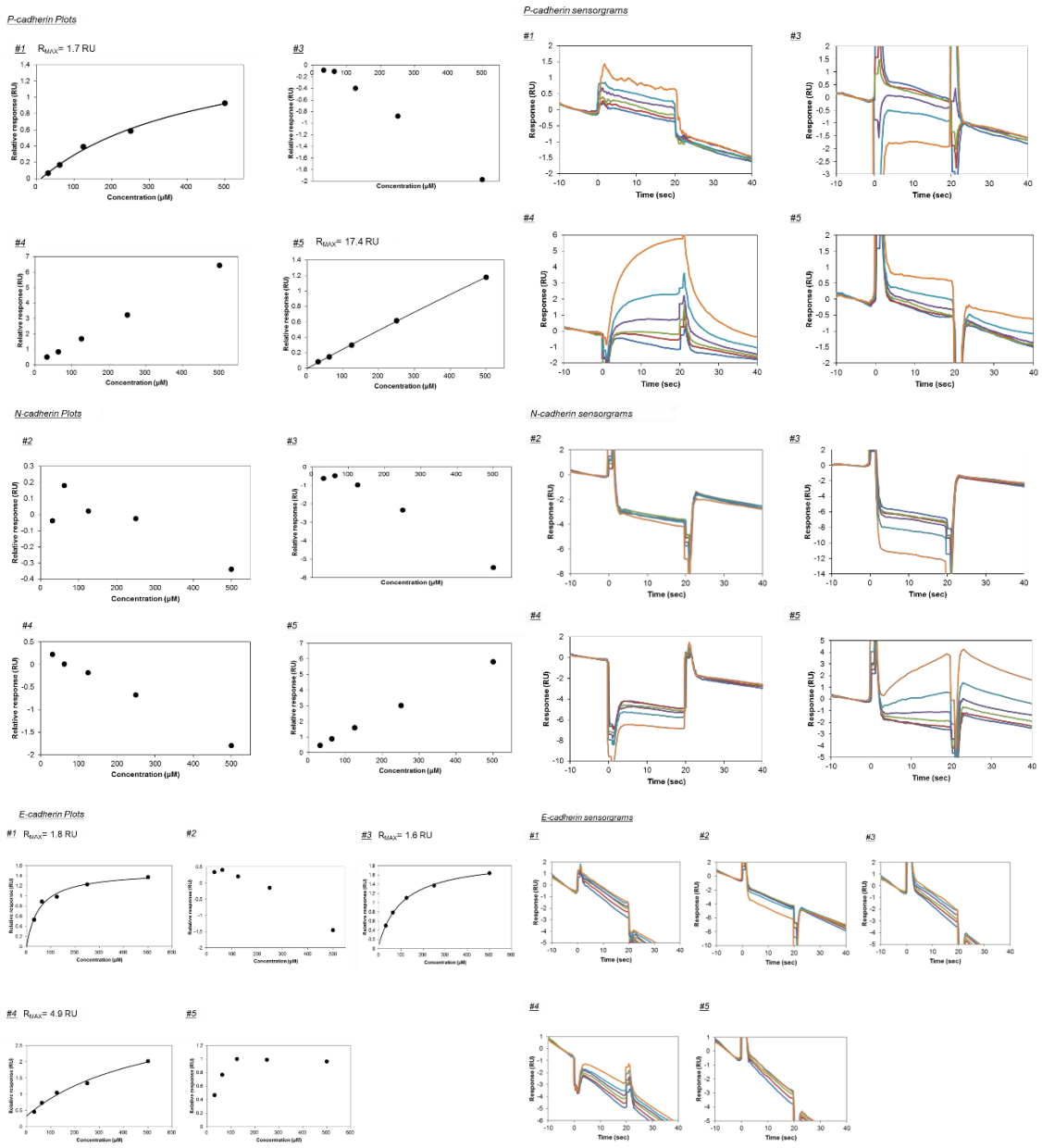

Fig. S2 Specificity check by SPR. Monomer construct (REC12) was immobilized on Sensor Chip SA via biotin-SA capture. Each compound was diluted from 500 μM to 31.2 μM with running buffer.

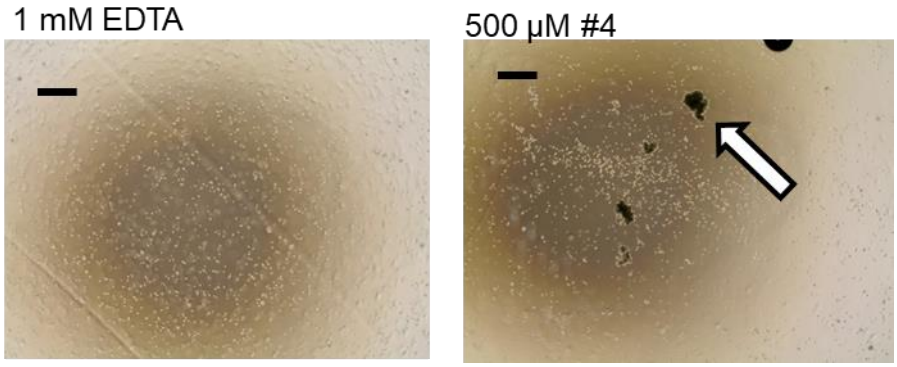

Fig. S3 Inhibition of forming aggregates by EDTA and aggregation formed by cells in the presence of compound #4. Cell aggregates were prepared by incubating cell samples for 30 minutes in the presence of 1 mM Ca^{2+} and 2 % DMSO. Images were taken after 30 minutes incubation.

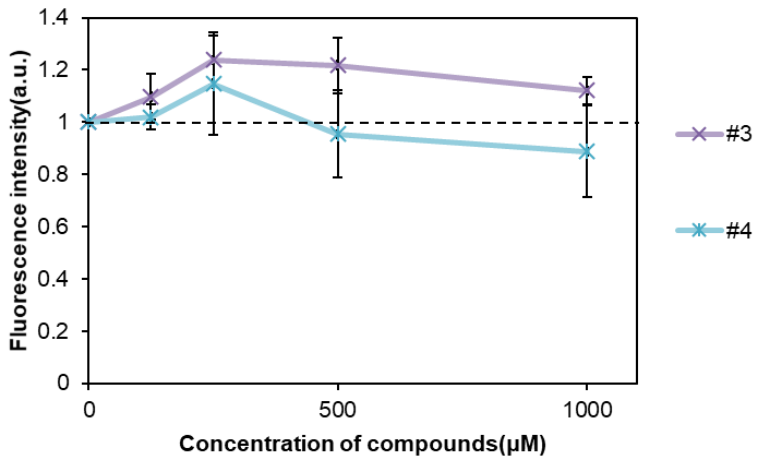

Fig. S4 Changes of fluorescence intensity with not hit compounds in cell adhesion assay.