

Identification of claudin-4 binder that attenuates tight junction barrier function by TR-FRET-based screening assay

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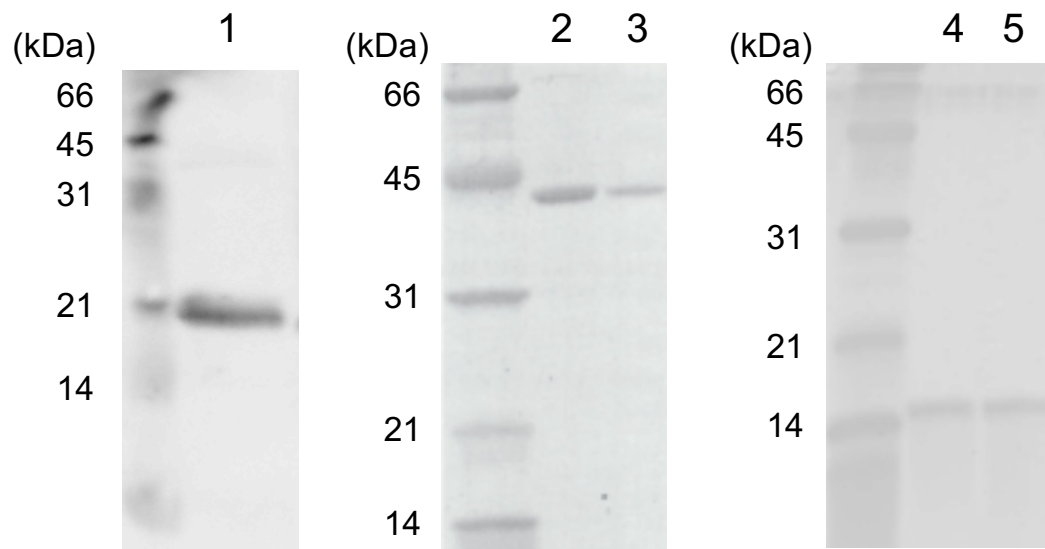
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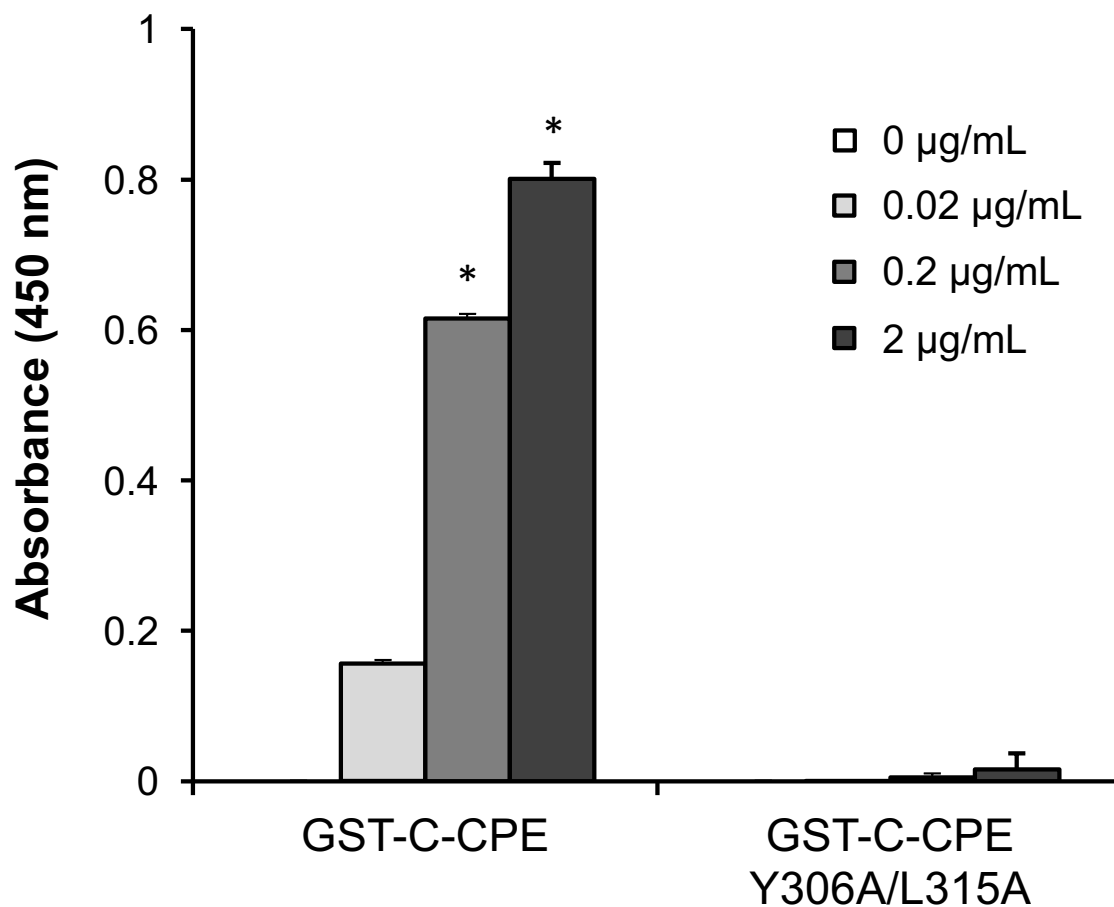
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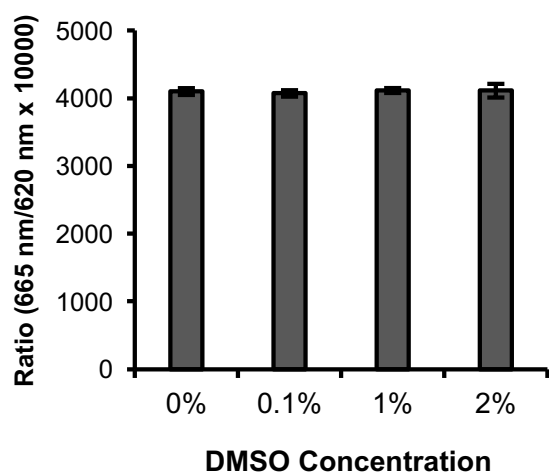
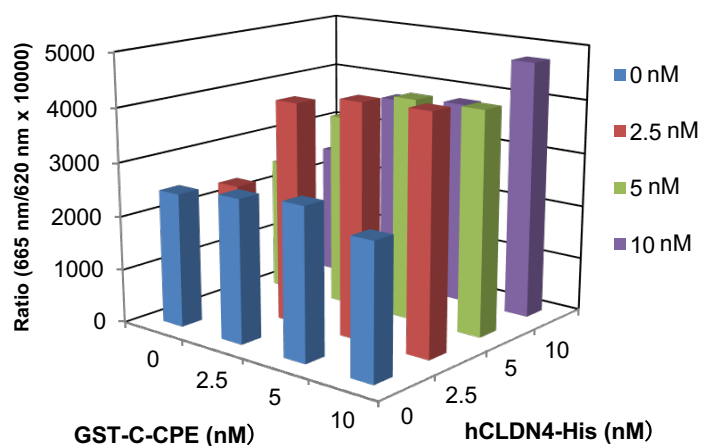
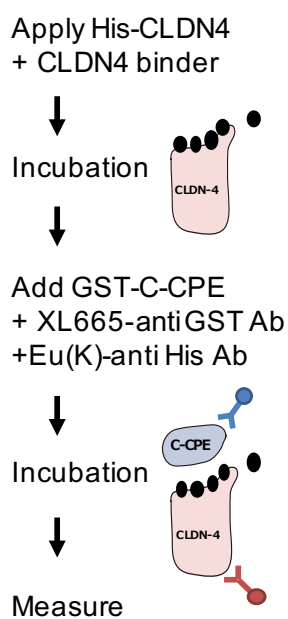
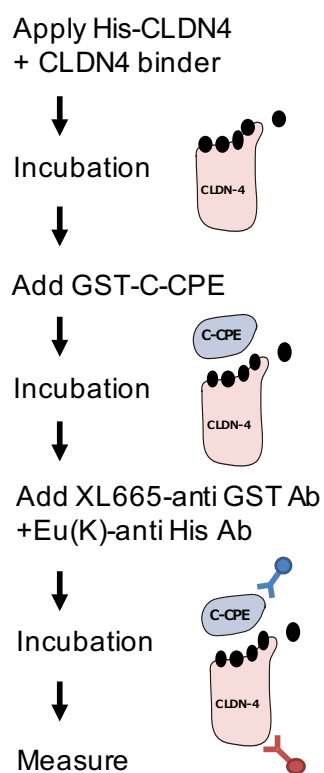
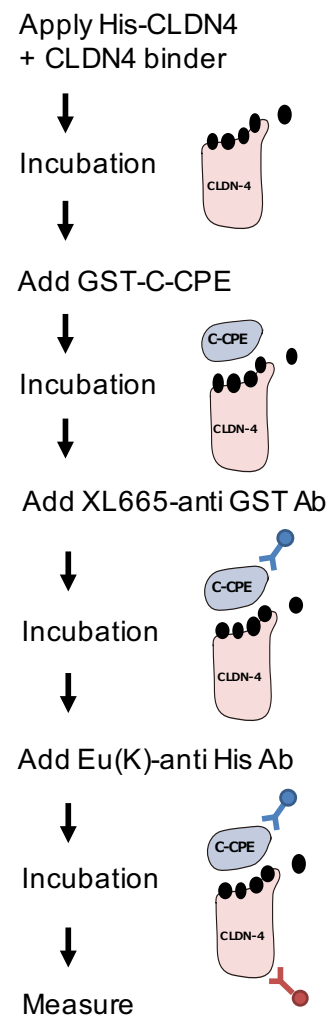
Supplementary Figure 1 Preparation of His-claudin-4, GST-C-CPEs, and C-CPEs.

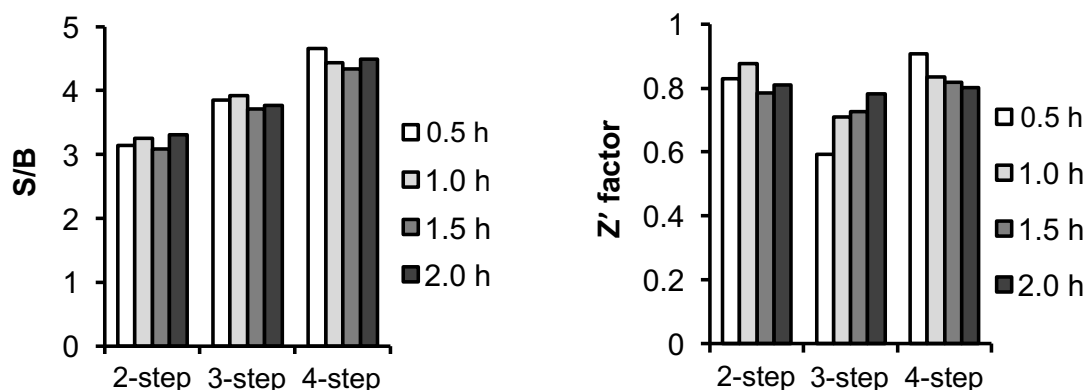
His-claudin-4, GST-C-CPEs and C-CPEs were prepared as described in the Methods.

Purification was confirmed by SDS-PAGE (0.5 µg/lane), followed by staining with Coomassie Brilliant Blue. Lane 1, His-claudin-4; lane 2, GST-C-CPE; lane 3, GST-C-CPE Y306A/L315A; lane 4, C-CPE; lane 5, C-CPE Y306A/L315A.



Supplementary Figure 2 ELISA-based binding assay for detection of His-claudin-4 and GST-C-CPE interaction. Plates were coated with His-claudin-4, after which GST-C-CPE or GST-C-CPE Y306A/L315A was added at the indicated concentration. The interactions of His-claudin-4 with GST-C-CPE or GST-C-CPE Y306A/L315A were detected by ELISA analysis. Data are means $\pm$ 1 SD ($n = 3$). *, $P < 0.05$ vs vehicle-treated group, as determined by using Dunnett's test.

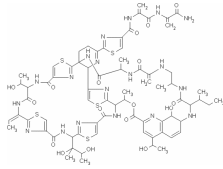
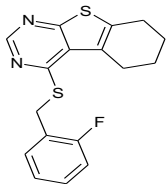
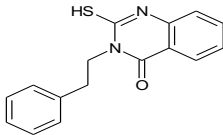
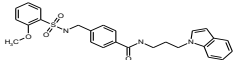
a**b****c****2-step assay****3-step assay****4-step assay**

d

Supplementary Figure 3 Assay optimisation for the claudin-4-C-CPE TR-FRET.

(a) DMSO tolerability of the claudin-4-C-CPE TR-FRET assay. His-claudin-4 (10 nM), GST-C-CPE (10 nM), Eu(K)-anti-His Ab (0.125 nM), XL665-anti-GST Ab (10 nM) and DMSO (final: 0%, 0.1%, 1%, 2%) were dissolved in TR-FRET buffer. After incubation for 30 min, FRET signals were measured. **(b)** Optimal concentrations of His-claudin-4 and GST-C-CPE in the TR-FRET assay. GST-C-CPE (0, 2.5, 5 or 10 nM) and His-claudin-4 (0, 2.5, 5 or 10 nM) with Eu(K)-anti-His Ab (0.125 nM) and XL665-anti-GST Ab (10 nM) were dissolved in TR-FRET buffer. After incubation for 30 min, FRET signals were measured. **(c)** Procedure for each type of assay. In the 2-step assay, His-claudin-4 (shown as pink shapes, with black ovals as claudin-4 binder) was incubated with GST-C-CPE (blue shapes), Eu(K)-anti His Ab (red), and XL665-anti GST Ab (blue). In the 3-step assay, His-claudin-4 was incubated first with GST-C-CPE, after which Eu(K)-anti His Ab and XL665-anti GST Ab were added concurrently. In the 4-step assay, His-claudin-4 was incubated with GST-C-CPE, after which XL665-anti GST Ab and Eu(K)-anti His Ab were added sequentially. **(d)** For each step of the assay, the mean signal-to-background (S/B) ratio at the indicated final reaction time (0.5, 1, 1.5 or 2 h) was calculated as the average signal ratio divided by the average signal ratio for 0 nM GST-C-CPE. The Z' factor at the indicated reaction time (0.5, 1, 1.5 or 2 h) was calculated by using the following formula: $1 - (3 \times \text{SD of each signal ratio} + 3 \times \text{SD of 0 nM GST-CPE [negative control] signal ratio}) / (\text{mean of each signal ratio} - \text{mean of negative control signal ratio})$. TR-FRET signals were measured by using an ARTEMIS plate reader at 620 and 665 nm emission.

Supplementary Figure 4

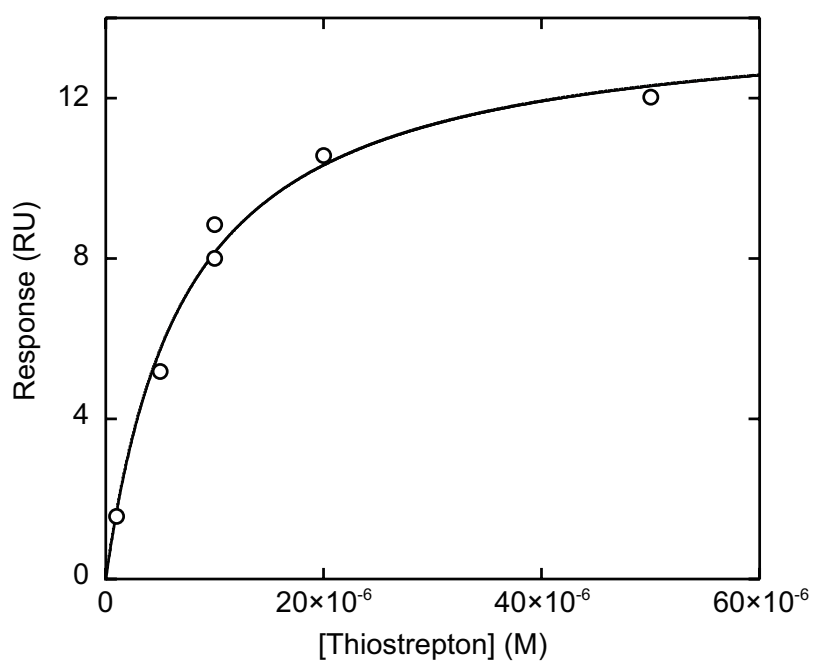
Compound	Structure	TR-FRET (% of control) ^b	Biacore analysis (RU) ^c	TER (%) ^d
Thiostrepton		66.24	32.1	55.2
Z20226792 ^a		70.07	15.2	72.5
Z55663919 ^a		62.43	3.4	70.7
Z32811226 ^a		63.75	4.8	90.1

^a These compounds were purchased from Enamine.

^b Inhibition of Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) signal by interaction between claudin-4 and C-CPE.

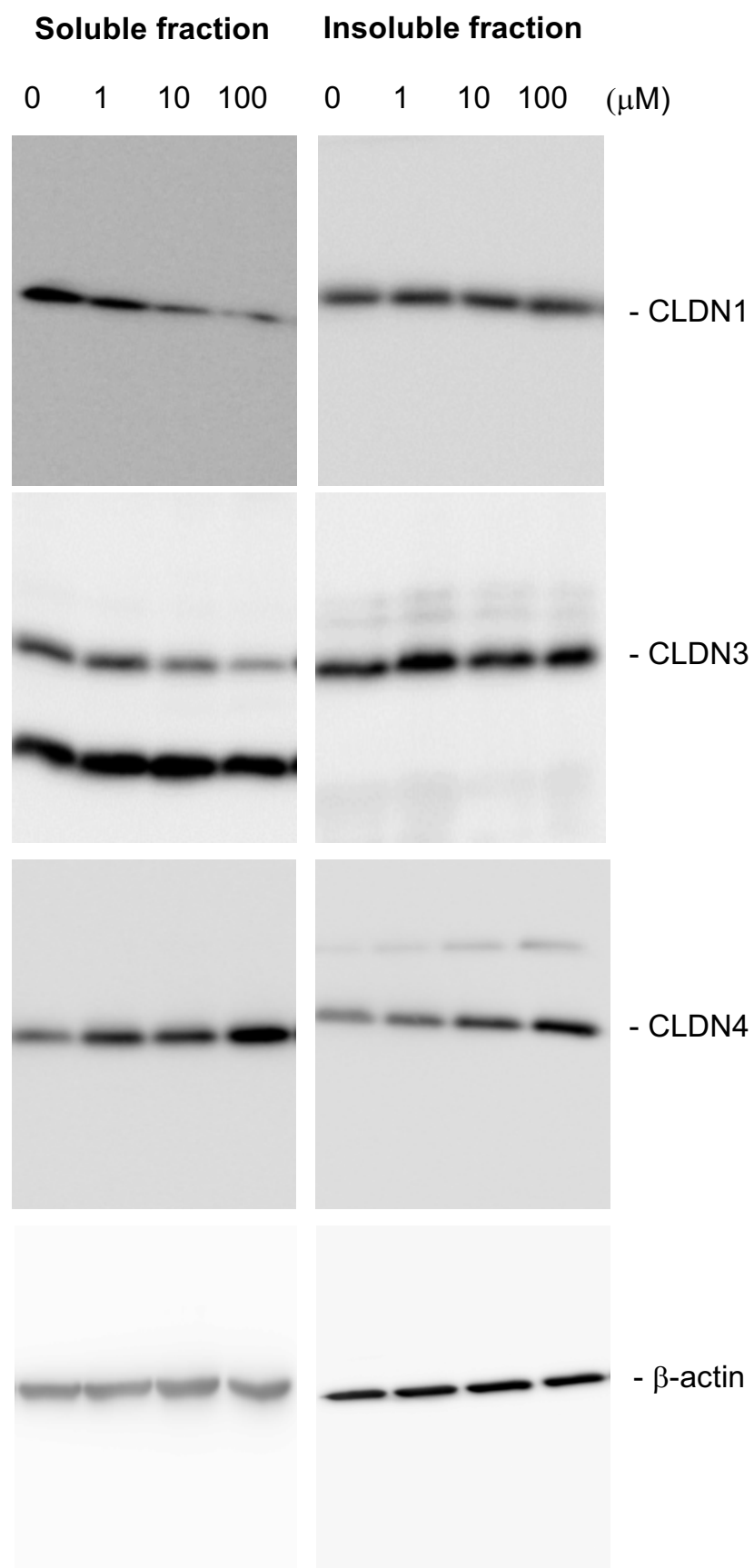
^c Validation of interaction with claudin-4 by biacore analysis. RU, Resonance Units.

^d The Caco-2 cells monolayers were treated with each compound (10 μ M) for 24 h. Transepithelial electrical resistance (TER) values are shown as percentages of the TER values at 0 h.

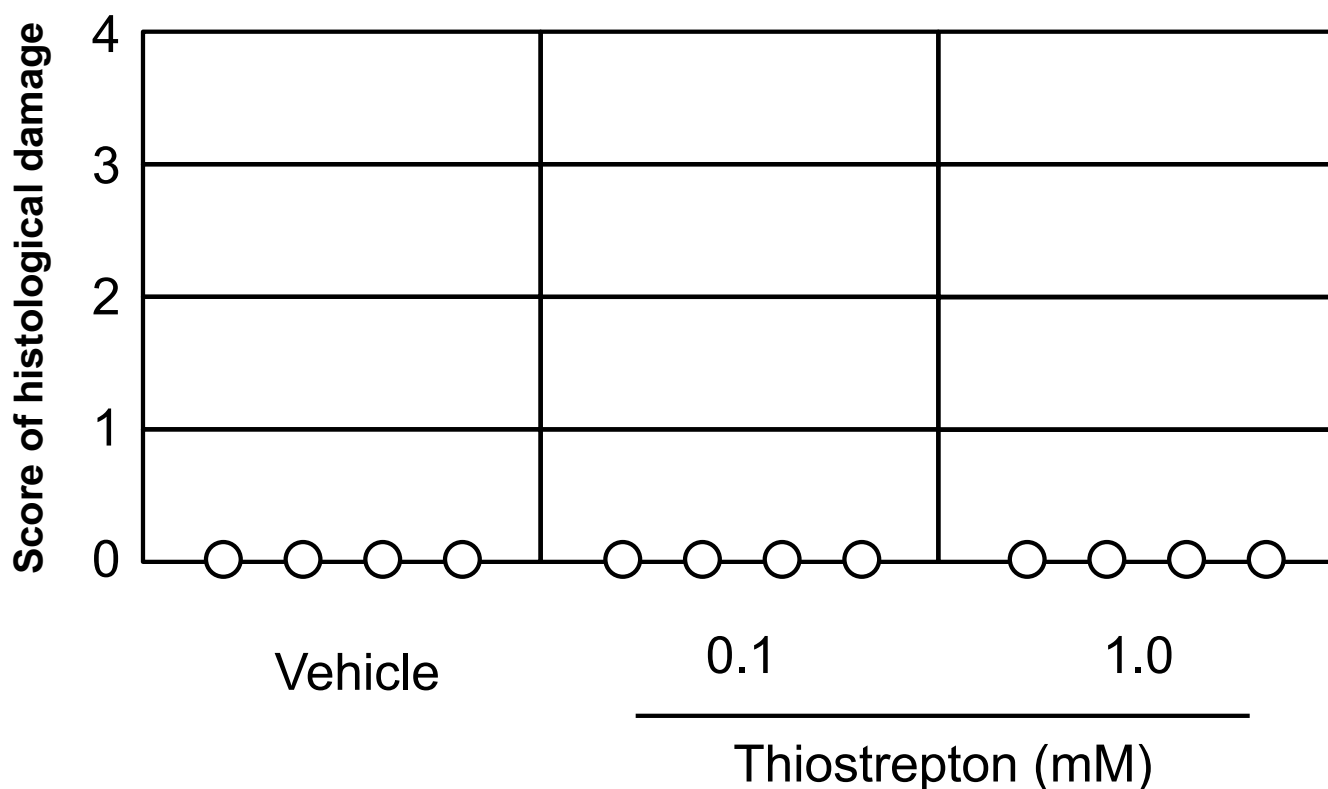


Supplementary Figure 5 Equilibrium analysis of claudin-4 and thioestrepton.

Several concentrations of thioestrepton were plotted against $R_{\max}$ values, and a sigmoid curve was fitted to estimate the dissociation constant (K_D).



Supplementary Figure 6 Full-length blots for Figure 4a.



Supplementary Figure 7 Scoring of histological damage of rat jejunum treated with thiostrepton or left untreated. Score 0, normal appearance; Score 1, analysed intestinal tissue showed very slight intraluminal bleeding, erosion, ulceration, stasis, inflammatory cell infiltration, goblet cell reduction, oedema or fibrin-like precipitate; Score 2, analysed intestinal tissue showed slight intraluminal bleeding, erosion, ulceration, stasis, inflammatory cell infiltration, goblet cell reduction, oedema or fibrin-like precipitate; Score 3, analysed intestinal tissue showed moderate intraluminal bleeding, erosion, ulceration, stasis, inflammatory cell infiltration, goblet cell reduction, oedema or fibrin-like precipitate; Score 4, analysed intestinal tissue showed marked intraluminal bleeding, erosion, ulceration, stasis, inflammatory cell infiltration, goblet cell reduction, oedema or fibrin-like precipitate.