Supporting Information

1 # Drug repurposing of itraconazole and estradiol benzoate against COVID-19 by
2 blocking SARS-CoV-2 spike protein-mediated membrane fusion

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9 The file includes:

10 Figures. S1 to S5.

11 Materials and Methods.

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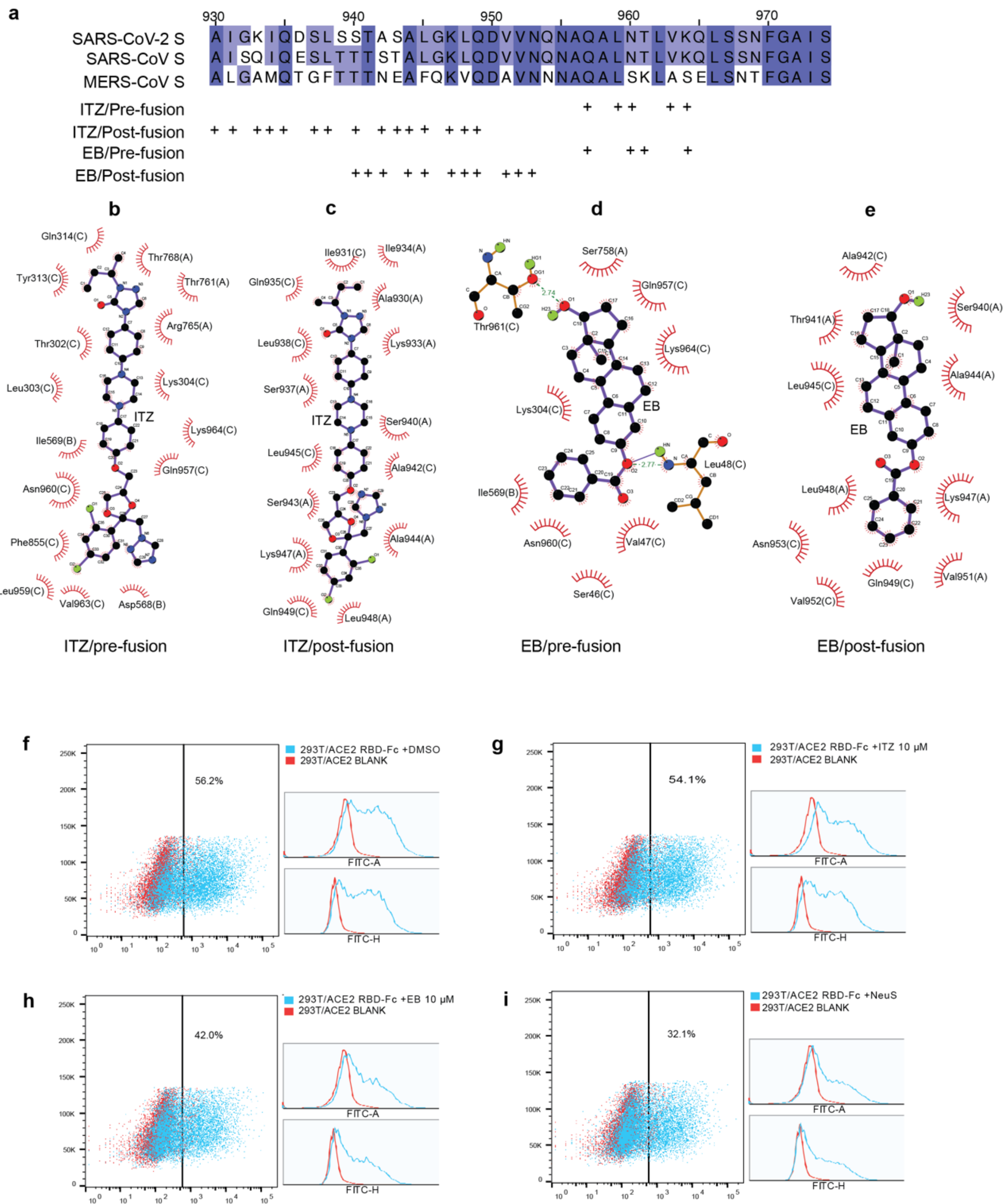
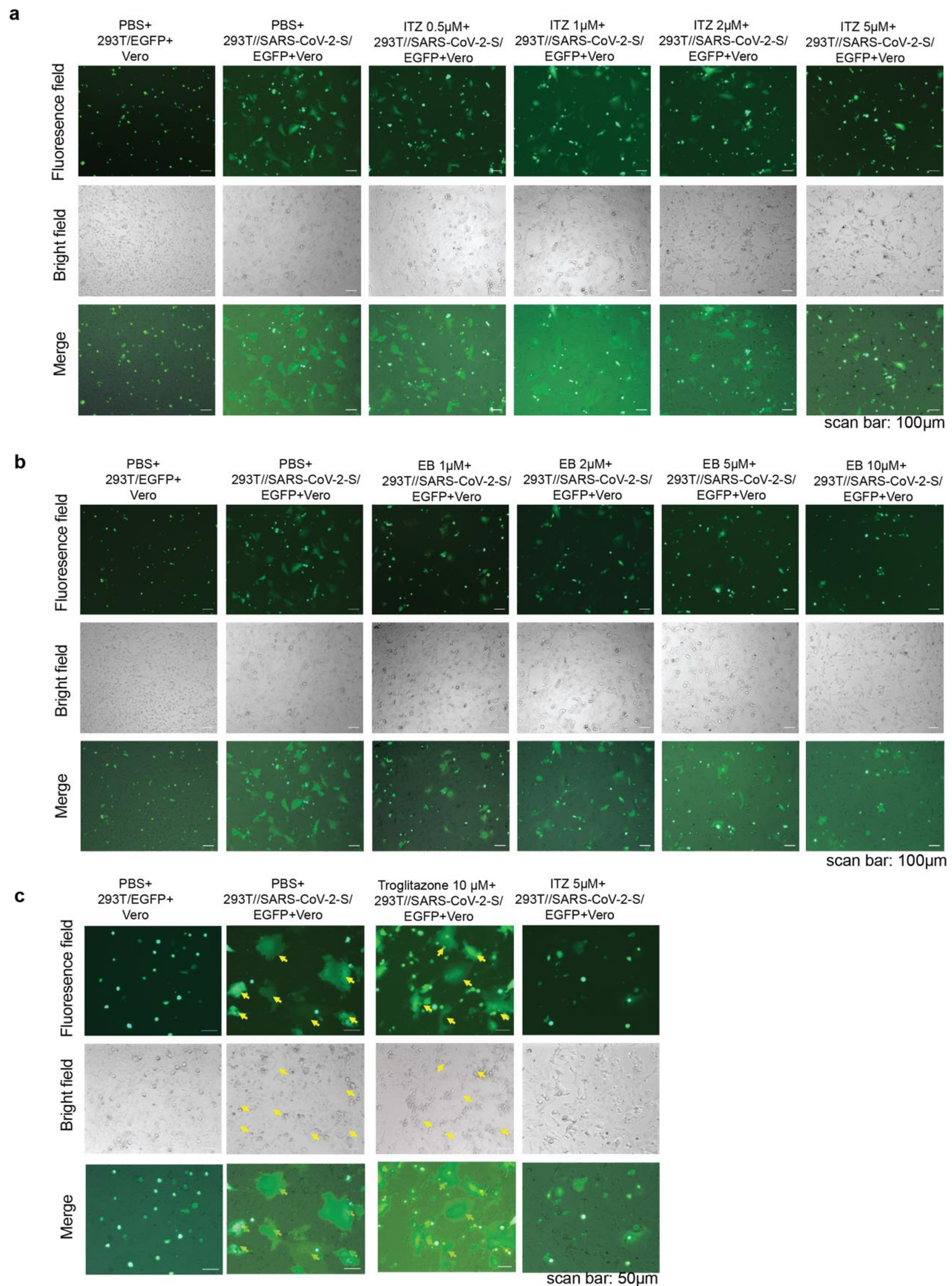


13 **Figure S1.** Binding details of ITZ/EB with pre-fusion and post-fusion S structures. a)
14 Sequence alignment of S protein HR1 region between residue 930 to residue 974
15 between SARS-CoV-2, SARS-CoV and MERS-CoV. “+” indicated the corresponding
16 residue involved in the interatction with ITZ or EB. b) Interaction details between ITZ
17 and pre-fusion S structure. c) Interaction details between ITZ and post-fusion S
18 structure. d) Interaction details between EB and pre-fusion S structure. e) Interaction

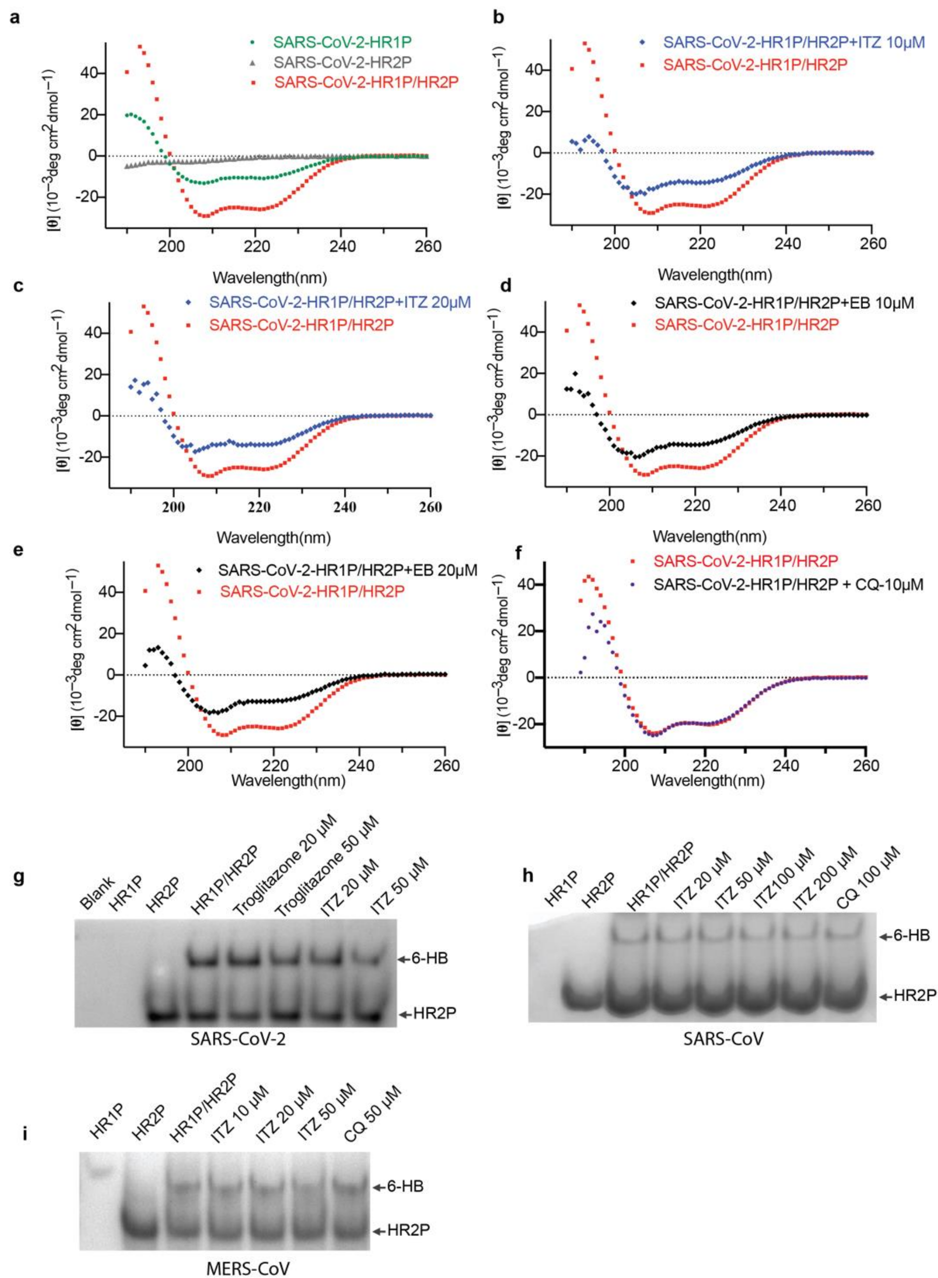
19 details between EB and post-fusion S structure. The figures are prepared by program
20 LigPlot, hydrophobic contacts are represented by eyebrow, hydrogen bonds are
21 represented by dotted lines. **f-i)** Flow cytometry analysis the binding of SARS-CoV-2
22 RBD to hACE2 receptor. Mock-incubated 293T-ACE2 cells (red) were used as
23 control. f) Fc-fused SARS-CoV-2 RBD protein (RBD-Fc, blue) bound strongly to
24 293T-ACE2 cells. g) After treatment with 10μM of ITZ (blue), the positive signals
25 for hFc (FITC) showed no difference with control group (red). h) The positive signals
26 for hFc (FITC) of EB treated group (blue). Experiments were repeated twice and
27 yielded similar results. i) The binding between SARS-CoV-2 RBD and cell-associated
28 hACE2 receptor could be significantly blocked by neutralizing serum (NeuS, blue).
29



30 **Figure S2.** Images of ITZ or EB inhibiting SARS-CoV-2 S-mediated cell-cell fusion.
31 a,b) Schematic representation of SARS-CoV-2 S-mediated cell-cell fusion after
32 treatment with gradient concentrations of ITZ (a) or EB (b) for 24 h. Representative
33 results were shown from one field selected randomly each sample with scale bars of

34 100 μm. c) Troglitazone was used as negative control in cell-cell fusion test.

35 Troglitazone showed no effects on spike-mediated cell-cell fusion.



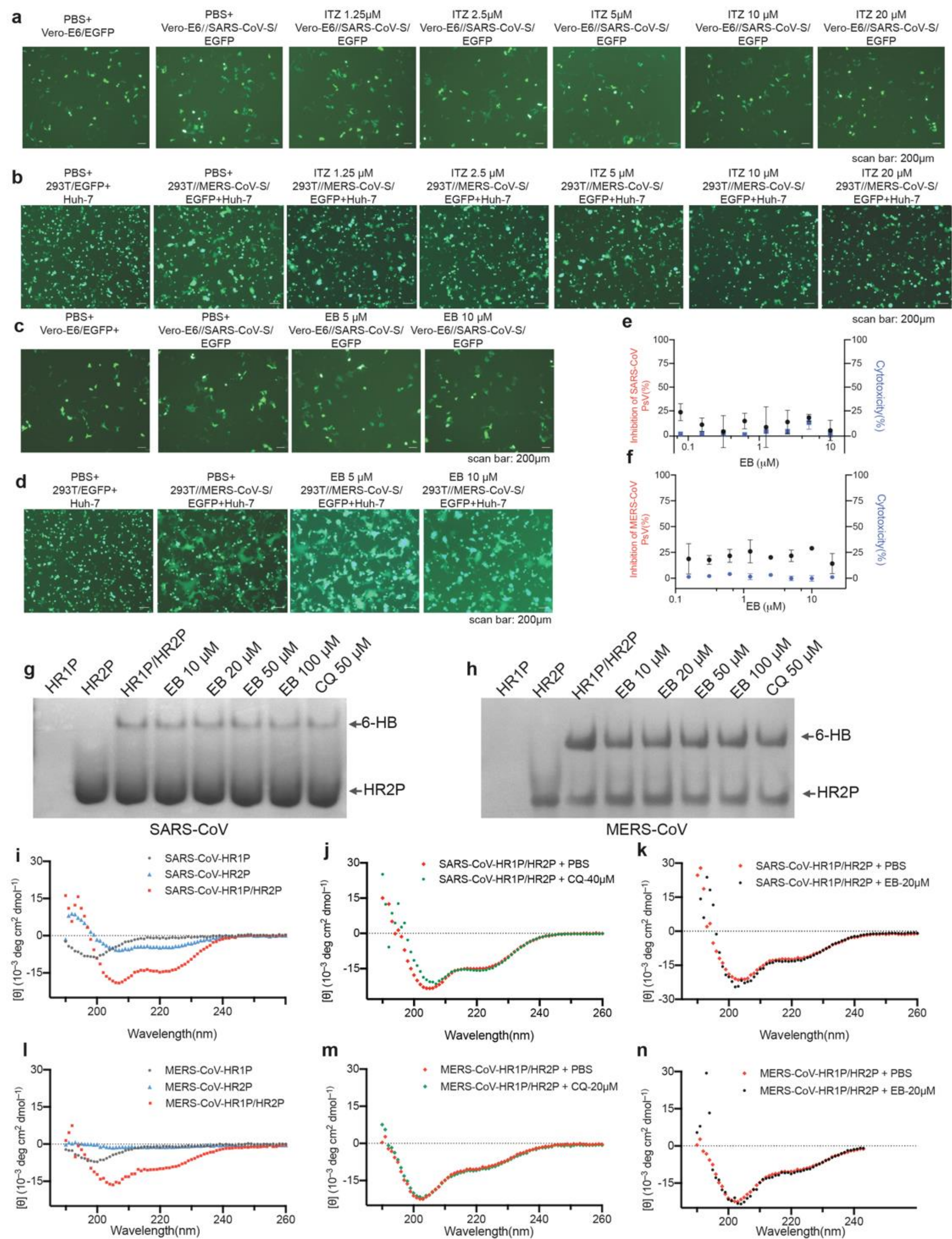
36 **Figure S3.** ITZ and EB reduced the formation of 6-HB. a) For the untreated control,

37 HR1 peptide (HR1P, green) was incubated with 50 mM phosphate buffer (pH7.2) for

38 30 min, and HR2 peptide (HR2P, gray) was added for a 30 min co-incubation. The

39 CD profile of HR1P/HR2P mixture is indicated in red, as determined by CD

40 spectroscopy, with the final concentration of each peptide being 10 μM. b-e, f) For the
41 experimental groups, HR1P was treated with indicated concentrations of ITZ (b,c),
42 EB (d,e) or CQ (f) for 30 min, and the CD profile of HR1P/HR2P and ITZ or EB
43 mixture showed the lower α-helical. The experiments were performed three times, and
44 the representative data were shown. g) N-PAGE assays for troglitazone and ITZ
45 treatment with 20 μM or 50 μM to test the disrupting the formtation of 6-HB. h, i)
46 Dose-dependent inhibition of SARS-CoV 6-HB formation (h) and MERS-CoV 6-HB
47 formation (i) by ITZ, as assesed by N-PAGE. SARS-CoV HR1R and SARS-CoV
48 HR2P were mixed at equimolar concentration (50 μM) in the presence of ITZ at 0, 20,
49 50, 100 and 200 μM. MERS-CoV HR1P (35μM) were prtreated with indicated
50 concentration of ITZ, followed by the addition of HR2P (35μM) and loading to the
51 gel. CQ (50 μM) was used as the negative control.



52 **Figure S4.** ITZ or EB blocked SARS-CoV S and MERS-CoV S by targeting to 6-HB.

53 a) Fluorescence images of ITZ inhibiting SARS-CoV S protein-mediated syncytium

54 formation. Vero-E6 cells transiently expressed SARS-CoV S protein and EGFP

55 (293T//SARS-CoV-S/EGFP). Then representative GFP-positive syncytia were

56 recorded following the description in the *Methods*, scale bars = 200 μm. b)

57 Fluorescence images of ITZ inhibiting MERS-CoV S protein-mediated syncytium

58 formation. 293T cells transiently expressed MERS-CoV S protein and EGFP
59 (293T//MERS-CoV-S/EGFP) as effecter cells, while Huh-7 cells as host cells.
60 Representative GFP-positive syncytia were recorded after treatment with ITZ. c)
61 Fluorescence images of EB inhibiting SARS-CoV S protein-mediated syncytium
62 formation. Vero-E6 cells transiently expressed SARS-CoV S protein and EGFP
63 (Vero-E6//SARS-CoV-S/EGFP). Then representative GFP-positive syncytia were
64 recorded following the description in the *Methods*, scale bars = 200 μm. d)
65 Fluorescence images of EB inhibiting MERS-CoV S protein-mediated syncytium
66 formation. 293T cells transiently expressed MERS-CoV S protein and EGFP
67 (293T//MERS-CoV-S/EGFP) as effecter cells, while Huh-7 cells as host cells.
68 Representative GFP-positive syncytia were recorded after treatment with EB. Three
69 images per condition were acquired and processed with scale bars of 200 μm.
70 Different EB concentration treatment to inhibit SARS-CoV pseduovirus assay (e) or
71 MERS-CoV pseduovirus assay(f). N-PAGE assay detection of EB has no effect on
72 SARS-CoV 6-HB formation (g) or MERS-CoV 6-HB formation (h). CD profiles for
73 SARS-CoV 6-HB (i), MERS-CoV 6-HB (l). j,m) 20 or 40 μM CQ were used for
74 negative control. k,n) EB (20 μM) treatment shows no effect on the formation of
75 SARS-CoV 6-HB (k) and MERS-CoV 6-HB (n).

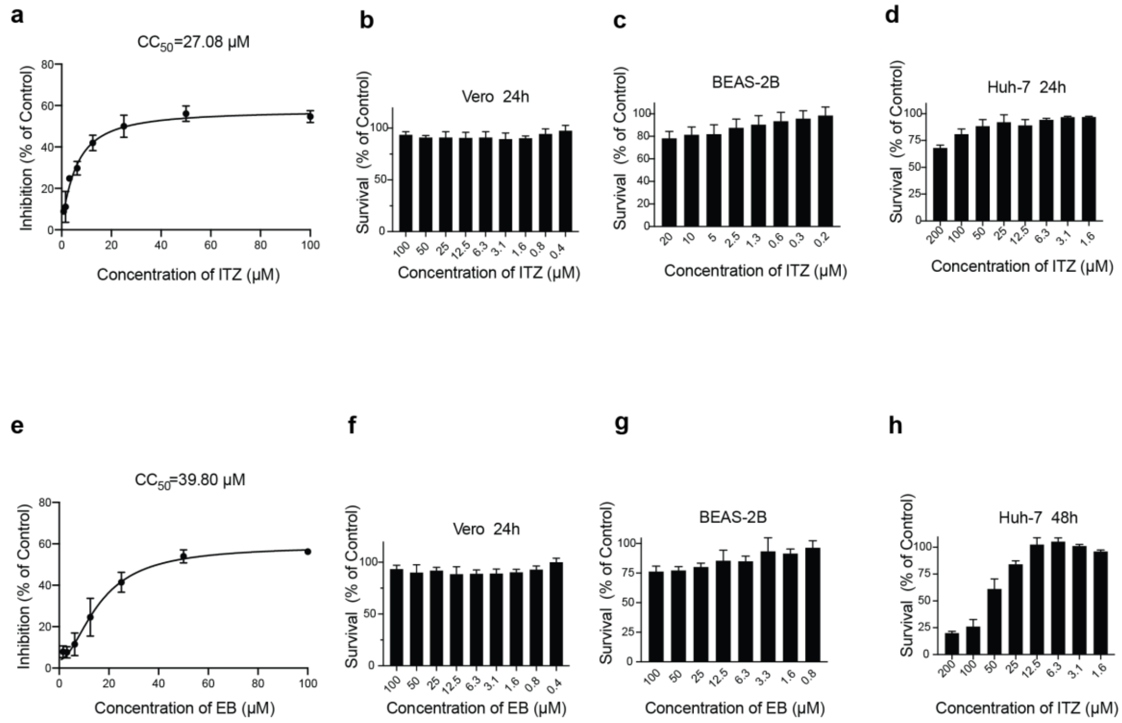


Figure S5. Cytotoxic effects of the ITZ and EB on different cell lines. a-d, h) Cytotoxicity of ITZ, which was detected on 293T-ACE2 (a), Vero (b), BEAS-2B (c) and Huh-7 (d, h) cells. e-g) Cytotoxic effects of EB on 293T-ACE2 (e), Vero (f) and BEAS-2B (g) cells. Data represent mean of independent biological replicates, n=3. Error bars: SD.

82 **Materials and Methods**

83 *Cell lines, drugs and peptides:* HEK-293T (Human, embryonic kidney), Vero
84 (African green monkey, kidney) , Vero-E6 (African green monkey, kidney) and Huh-7
85 (Human, liver) cells were cultivated in Dulbecco's Modified Eagle Medium (Gibco)
86 supplemented with 10% fetal bovine serum (Capricorn Scientific), 100 U/mL of
87 penicillin and 0.1 μg/mL of streptomycin (Gibco). HEK-293T cells that stably express
88 human ACE2 (293T-ACE2) have been described previously[1] and were cultivated in
89 the presence of 2 μg/mL puromycin (Invivogen). BEAS-2B (Human, epithelial cells
90 were isolated from normal bronchial epithelium) was cultured in Lonza BEGM
91 BulletKit (Lonza). All cell lines were obtained from ATCC (Manassas, VA) and were
92 incubated at 37 ℃ and 5% CO_2 in a humidified atmosphere. ITZ and EB were
93 purchased from TargetMol (China). The peptides, including SARS-CoV-2 HR1P
94 (residues 924-965), SARS-CoV-2 HR2P (residues 1,168-1,203), SARS-CoV HR1P
95 (residues 892-931), SARS-CoV HR2P (residues 1,153-1,189), MERS-CoV HR1P
96 (residues 998-1,039) and MERS-CoV HR2P (residues 1,251-1,286) were synthessized
97 by GL Biochem Ltd (China).

98 Plasmids encoding S glycoprotein (pAAV-IRES-EGFP-SARS-CoV-2 S,
99 pAAV-IRES-EGFP-SARS-CoV S, pAAV-IRES-EGFP-MERS-CoV S) have been
100 described previously. Plasmid pAAV-IRES-EGFP was purchased from Hedgehogbio
101 Science and Technology Ltd.

102 *Ligand-protein interaction analysis:*For each drug docking, ten binding modes were
103 analyzed and the pose with top score was selected according to the binding affinity

104 from computation, and further manually checked the interaction. The detailed
105 interaction between ITZ or EB and pre-fusion or post-fusion S were made by ligplus.
106 *Flow cytometry assay:* SARS-CoV-2 RBD-Fc protein was expressed and purified
107 from 293F cell. Flow cytometry analysis was carried out as described previously.
108 Briefly, the 293T-ACE2 cells were plated in 12-well plates overnight. RBD-Fc protein
109 (2 μg/ml) were pre-incubated with different concentration of ITZ or EB at room
110 temperature for 30 min. The mixture was added to 293T-ACE2 cells and incubation at
111 37℃ for 20 min. After staining with goat anti-human FITC-conjugated IgG antibody
112 (1:500, Sigma), the cells were analyzed by flow cytometer (BD FACSCanto II) and
113 Flowjo software.
114 *Cell-cell fusion assay:* To prepare effector cells (293T//SARS-CoV-2-S/EGFP),
115 HEK-293T cells were transfected with pAAV-IRES-GFP-SARS-CoV-2 S or vehicle
116 pAAV-IRES-GFP for 48 h with PolyJet (SignaGen) transfection. Target cells (Vero)
117 were seeded in 96-well plates (10^4 cells for each well) 4 h prior to cell-cell fusion
118 assay. 293T//SARS-CoV-2-S/EGFP cells were incubated with ITZ or EB at room
119 temperature for 30 min and were overlaid on Vero cells with 2×10^4 cells/well.
120 293T/EGFP cells were used as a negative control. After 24 h incubation, the samples
121 were visualized by bright-field and fluorescence-field microscopy using a Zeiss
122 scanner and the ZEN imaging software.
123 To prepare Vero-E6//SARS-CoV-S/EGFP cells as described before[2], Vero-E6 cells
124 were grown on coverslips in 24-well plates and transfected with
125 pAAV-IRES-GFP-SARS-CoV S or pAAV-IRES-GFP with PolyJet (SignaGen)

126 transfection. After 6 h, Vero-E6//SARS-CoV-S/EGFP cells were treated with gradient
127 concentrations of ITZ for 24 h. Cells were incubated for 1 hour in serum-free medium
128 containing of 2μg/mL trypsin to induce fusion. The medium were replaced with fresh
129 DMEM containing 10% FBS, and the cells were incubated for 6 h. Vero-E6/EGFP
130 cells were used as a negative control. The samples were visualized by bright-field and
131 fluorescence-field microscopy using a Zeiss scanner.

132 To prepare effector cells (293T//MERS-CoV-S/EGFP), HEK-293T cells were
133 transfected with pAAV-IRES-GFP-MERS-CoV S or pAAV-IRES-GFP for 48 h with
134 PolyJet (SignaGen) transfection. After Huh-7 cells were co-cultured with
135 293T//MERS-CoV-S/EGFP or 293T/EGFP cells in the absence or presence of ITZ or
136 EB for 48 h, cell-cell fusion were photographed under a Zeiss microscope with
137 fluorescence or bright light.

138 *Native-PAGE (N-PAGE)*: N-PAGE was used to detect the inhibitory activity of ITZ
139 or EB on formation of 6-HB between HR1P and HR2P (SARS-CoV-2/ SARS-CoV /
140 MARS-CoV) as described previously. HR1P was dissolved in ddH_2O, and HR2P was
141 dissolved in phosphate buffer (pH 7.4). HR1P (30 μM for SARS-CoV-2/ 50 μM for
142 SARS-CoV / 35 μM for MARS-CoV) with or without indicated concentration of ITZ
143 or EB were incubated at room temperature for 30 min, followed by the addition of
144 HR2P (30 μM for SARS-CoV-2/ 50 μM for SARS-CoV / 35 μM for MARS-CoV).
145 The mixtures were incubated for another 30 min and separated by 18% Tris-glycine
146 gel with constant 125V at room temperature for 2 h. The gel was stained with
147 coomassie blue staining (HaoMa Biotechnology) and imaged with a Tanon 2500-B

148 scanner (Tanon Science & Technology).

149 *Circular dichroism (CD) spectroscopy:* CD spectra were monitored on a Chirascan
150 plus ACD (Applied Photophysics Ltd). HR1P and HR2P (SARS-CoV-2/ SARS-CoV /
151 MARS-CoV) were dissolved in buffer (0.1M KCl, 0.05M PO_4, pH 7.2) at a final
152 concentration of 10 μM. Briefly, HR1P were incubated with PBS or drugs at 25 °C for
153 30 min, followed by addition of HR2P (10 μM). After further incubation for 30 min,
154 the CD wave scans were measured from 190 to 260 nm at 4 °C with the bandwidth of
155 2 nm and the step size of 1nm. The $[\theta]_{222}$ value of -33,000 deg cm^2 $dmol^{-1}$ was taken
156 to correspond to 100% α-helical content.

157 *Cytotoxicity assay:* Cytotoxicity of drugs to cells (Vero-E6, Vero, Huh-7, 293T-ACE2,
158 BEAS-2B) was detected by Cell Counting Kit-8 (CCK-8) (Topscience). Briefly, cells
159 were seeded into 96-well plates (10^4 per well) and cultured at 37 °C overnight. A total
160 100 μl of DMEM containing indicated concentrations of ITZ or EB was added to cells.
161 Following 24, 48 or 72 h incubation, 10 μl of CCK-8 solution was dropped to each
162 well 4 h prior to the measurement of absorbance at 450 nm.