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Electronic Supplementary Material

Griffithsin with A Broad-Spectrum Antiviral Activity by Binding Glycans in Viral Glycoprotein Exhibits Strong Synergistic Effect in Combination with A Pan-Coronavirus Fusion Inhibitor Targeting SARS-CoV-2 Spike S2 Subunit

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Cells, viruses, proteins and plasmids

HEK-293T (CRL-3216^{TM}) and Vero E6 (CRL-1586^{TM}) cells were obtained from the American Type Culture Collection (ATCC). HuH-7 cells were obtained from the Cell Bank of the Chinese Academy of Science and 293T/ACE2 cells were kindly provided by Lanying Du. All of these cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, USA). Fc-tagged SARS-CoV-2 RBD (COV-VM5BD), Fc-tagged SARS-CoV-2 S1 protein (COV-VM5S1) and His-tagged human ACE2 (ACE-HM401) were obtained from Kactus Biosystems. A codon-optimized gene encoding griffithsin was synthesized by the Beijing Genomics Institution and cloned into pET-28a vector. SARS-CoV-2 S protein-expressing plasmid (pcDNA3.1-SARS-CoV-2-S and pAAV-SARS-CoV-2-S-IRES-GFP) and the luciferase reporter vector (pNL4-3.Luc.R-E-) were stored in our laboratory. Griffithsin was expressed in the *E. coli* BL21 (DE3) transformed with pET-28a-Griffithsin and purified by nickel column. SARS-CoV-2 strain nCoV-SH01 was propagated in Vero E6 cells and maintained in a biosafety level 3 facility of Fudan University.

Production of pseudotyped SARS-CoV-2

Lentivirus-based pseudovirus of SARS-CoV-2 was produced as previously described (Xia *et al*. 2020). Briefly, HEK-293T cells were transfected with pNL4-3.Luc.R-E- and pcDNA3.1-SARS-CoV-2-S using Vigofect (Vigorous Biotechnology, China). SARS-CoV-2 pseudoviruses in the supernatant were harvested at 72 h after transfection. Pseudotyped viruses were stored at -80℃ without repeated freeze-thaw cycles.

Dose-response effect of griffithsin on SARS-CoV-2 pseudovirus infection

A total of 1×10^4 HuH-7 cells per well were seeded into a 96-well plate and cultured for 24 h. Griffithsin, EK1 and bovine serum albumin (BSA) were serially diluted and added to SARS-CoV-2 pseudovirus with an equal volume at 37℃ for 30 min. The mixtures were added to the HuH-7 cells in the 96-well plate. After incubation of 12 h, the mixtures were replaced by fresh medium and HuH-7 cells were cultured for

48 h. Luciferase assay was conducted to assess the inhibitory effect of griffithsin on SARS-CoV-2 pseudovirus infection.

Inhibition of SARS-CoV-2 S-mediated cell-cell fusion

Cell-cell fusion inhibition assay was performed as reported previously (Xia et al. 2020). Briefly, HuH-7 cells were seeded into a 96-well plate and cultured for 5 h. HEK-293T cells expressing SARS-CoV-2 S/GFP were added in the presence of griffithsin, EK1 and BSA at the indicated concentrations and cultured for 2 h, while cells treated with phosphate-buffered saline (PBS) were used as a negative control. Four fields were randomly selected using fluorescence microscopy and the fusion rate was calculated.

Inhibition of live SARS-CoV-2 infection

A total of 5×10^5/mL Vero E6 cells were seeded into 96-well plate one day prior to infection. GRFT were mixed with live SARS-CoV-2 (100 $TCID_{50}$) at 37℃ for 1 h and then added into Vero E6 cells. After incubation of 1 h, the supernatants were replaced by Dulbecco's Modified Eagle's Medium with penicillin (100 U/mL), streptomycin (100 mg/mL) and 10% fetal bovine serum (FBS, Gibco). After 48 h, supernatants were harvested and Vero E6 cells were fixed with PBS containing 4% paraformaldehyde and then treated with PBS containing 0.2% triton X-100. The inhibitory activity of GRFT against live SARS-CoV-2 were measured by immunofluorescence assay and quantitative reverse transcriptase PCR (qRT-PCR). SARS-CoV-2 RNA copies in the supernatants were detected by qRT-PCR. RNA in the supernatants were extracted using EasyPure Viral DNA/RNA Kit (TransGen Biotech, China), and quantified using One Step PrimeScript™ RT-PCR Kit (Takara, Japan) with probe (5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3', GENEWIZ). The forward primers (5'-GGGGAACTTCTCCTGCTAGAAT-3') and reverse primers (5'-CAGACATTTTGCTCTCAAGCTG-3') were obtained from GENEWIZ and used for the SARS-CoV-2 RNA amplification. The nucleocapsid of SARS-CoV-2 in Vero E6 cells were measured by immunofluorescence assay. Briefly, Vero E6 cells were blocked by PBST containing 1% bovine serum albumin (BSA) at 37℃ for 1 h and washed with PBST for 2 times. SARS-CoV-2 nucleocapsid antibody (1:200, Sino Biological) were added to Vero E6 cells. After incubation of 1 h, Vero E6 cells were washed with PBST for 3 times and the Alexa Fluor 488 goat anti-rabbit IgG (1:100, Thermo Fisher) were added to Vero E6 cells. After incubation of 1 h, Vero E6 cells were washed for 3 times and then DAPI were added to Vero E6 cells for 5 min. After washed for 3 times, Vero E6 cells were imaged on fluorescence microscope.

Time-of-addition assay

HuH-7 cells (1×10^4 cells/well) were seeded into a 96-well plate one day prior to infection. Griffithsin at a final concentration of 1000 nmol/L was added 0.5 h before or 0, 0.5, 1, 2, 4, 6, 8 h after SARS-CoV-2 pseudovirus infection. After incubation of 12 h, the mixtures were replaced by fresh medium and incubation continued for 48 h. Luciferase assay was performed to assess the inhibitory effect.

Time-of-removal assay

A total of 1×10^4 HuH-7 cells per well were seeded into a 96-well plate and cultured for 24 h. Griffithsin at a final concentration of 1000 nmol/L was added to HuH-7 cells for 1 h before SARS-CoV-2 pseudovirus infection. After washed 3 times with PBS, HuH-7 cells were cultured with SARS-CoV-2 pseudovirus, while HuH-7 cells cocultured with pseudovirus and griffithsin were used as a control. After incubation of 12 h, pseudoviruses were removed and HuH-7 cells were cultured with Dulbecco's Modified Eagle's Medium for 48 h. Luciferase assay was performed to assess the inhibitory effect.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay was performed to test the binding of griffithsin to S1 protein and RBD. A 96-well polystyrene plate (Corning, USA) was coated with GRFT or BSA (10 ug/mL) at 37℃

for 2 h. After washed 3 times with PBST, the plate was blocked with 2% (W/V) gelatin at 37℃ for 2 h. The plate was washed 3 times and incubated with indicated concentrations of S1 protein or RBD diluted in PBS at 37℃ for 1 h. After washed 3 times with PBST, the plate was incubated with a horseradish peroxidase-conjugated anti-human IgG antibody (Abcam) at a 1:5000 dilution at 37℃ for 1 h. The plate was washed and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added. The reaction was stopped with 2 mol/L H_2SO_4 and the absorbance was measured at 450 nm.

To test the inhibitory effect of mannose on the binding of griffithsin to SARS-CoV-2 S1 protein and RBD, a 96-well polystyrene plate was coated with S1 protein or RBD (2 μg/mL) and blocked with 2% gelatin for 2 h. After washed 3 times with PBST, the plate was incubated with griffithsin in the presence or absence of mannose at indicated concentrations. The plate was washed and incubated with anti-6His antibody. The plate was washed and TMB was added. After stopped with H_2SO_4, the plate was measured at 450 nm.

To test whether griffithsin could inhibit the binding of S1 protein and RBD to human ACE2, a 96-well polystyrene plate was coated with ACE2 protein (2 μg/mL) and blocked with 2% gelatin for 2 h. After washed 3 times with PBST, the plate was incubated with RBD (4 ng/mL) or S1 protein (200 ng/mL) in the presence of GRFT or ACE2 protein. The plate was washed and incubated with a horseradish peroxidase-conjugated anti-human IgG antibody (Abcam) at a 1:5000 dilution. After TMB was added, the reaction was stopped with H_2SO_4. The absorbance was measured at 450 nm.

Effect of GRFT on the binding of S1 protein and RBD to HEK-293T/ACE2 cells

A total of 5×10^5/mL HEK-293T/ACE2 cells and HEK-293T cells were seeded into 96-well plate and cultured for 24 h. HEK-293T/ACE2 cells and HEK-293T cells were fixed with PBS containing 4% paraformaldehyde for 10 min. After washed with PBST for 3 times, cells were blocked with PBST containing 2% gelatin for 1 h. S1 protein (1 μg/mL) and RBD (0.1 μg/mL) were incubated in the presence of GRFT or ACE2 for 1 h before added into HEK-293T/ACE2 cells, while HEK-293T cells treated with S1 protein or RBD were as a control. After incubation of 1 h, cells were washed with PBST for 3 times and FITC conjugated anti-human IgG antibody (1:100, Abcam) were added into 96-well plate for 1 h. After washed for 3 times, cells were incubated with DAPI for 5 min. Cells were washed and then imaged on fluorescence microscope.

Effect of mannose on GRFT-mediated inhibition of SARS-CoV-2 pseudovirus infection

HuH-7 cells were seeded to 96-well plate one day prior to infection. GRFT were incubated in the presence or absence of mannose at indicated concentrations for 2 h and then added into SARS-CoV-2 pseudoviruses. After incubation of 30 min, mixtures were added into HuH-7 cells. After incubation of 12 h, mixtures were removed and cells were cultured in fresh Dulbecco's Modified Eagle's Medium for 48 h. The inhibitory effect was measured by luciferase assay.

Synergy analysis

Griffithsin and EK1 were tested for SARS-CoV-2 pseudovirus infection inhibition individually and in combination at a fixed ratio. The combination index was calculated using the CalcuSyn program based on the median effect equation model of Chou and Talalay as previously described (Chou TC, 2006).

Reference

Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58:621–681.

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