Supporting Information

Ginkgolic acids inhibit SARS-CoV-2 and its variants by blocking the spike protein/ACE2 interplay

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1. Materials and methods

1.1. Protein expression and purification

The recombinant protein of the extracellular domain of human ACE2 fused with hIgG1-Fc (ACE2-Fc) was prepared as follows. The DNA fragment encoding ACE2 (residues 18–740) was subcloned into the mammalian expression vector pINFUSE (Vivogen, San Diego, CA, USA) with a hIgG1-Fc tag. The high-quality plasmids

were transfected into HEK293F cells (Shanghai Cell Line Bank, China) by PEI (Polysciences, Warrington, PA, USA). After five days of culture, the cell culture supernatant was harvested and purified by protein A. The cDNA of SARS-CoV-2 PL^{pro} and $3CL^{pro}$ were cloned into pET-22b and pET29a(+) vector with C-terminal 6×His tags, respectively, as previously reported [1, 2]. These two plasmids were then transformed into BL21 (DE3) cells for proteins expression and purified by Ni-NTA colums (GE Healthcare). As for SARS-CoV-2 RdRp, the full-length gene was cloned into a pFastBac baculovirus expression vector with C-terminal 8 × His tag as described previously [3]. SARS-CoV-2 RdRp was expressed in Spodoptera frugiperda (Sf9) cells and purified by Ni-NTA beads (GE Healthcare). Purified proteins were analyzed by SDS-PAGE to ensure purity and appropriate molecular weights.

1.2. FRET-base assay for SARS-CoV-2 PL^{pro} activity

A fluorescence resonance energy transfer (FRET) protease assay was applied to measure the inhibitory activity of compounds against the SARS-CoV-2 PL^{pro}. The fluorogenic substrare RLRGG-AMC was synthesized by GenScript (Nanjing, China). Firstly, 60 nM PL^{pro} was incubated with the indicated concentrations of the tested compounds in the condition of 50 mM HEPES, pH 7.5, 0.1 mg/mL BSA, 5 mM DTT for 30 min. The reactions were initiated by the addition of 20 μM fluorogenic peptide. After that, the fluorescence signal at 360 nm (excitation)/460 nm (emission) was measured immediately every 30s for 5 min with a Bio-Tek Synergy H1 plate reader (BioTek, Winooski, USA). The initial velocities of reactions with compounds added at various concentrations compared to the reaction added with DMSO were calculated.

1.3. Fluorescence-based assay for SARS-CoV-2 RdRp activity

The detection of RNA synthesis by SARS-CoV-2 RdRp was established with the fluorescent dye QuantiFluor® dsDNA System (Promega, Madison, WI, USA), which bound dsRNA but not ssRNA template molecules. The fluorescence emitted was recorded using a Bio-Tek Synergy H1 plate reader (BioTek, Winooski, USA) with excitation and emission filters at 504 and 531 nm, respectively. The assay recorded the synthesis of dsRNA in a reaction using a self-primer RNA as a template and ATP as the natural substrate, and the self-primer RNA was synthesized by Genscript Biotech Corporation with the sequences of 5'-bio-UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUAACAGGUUCUAGAACCUGUU-3'. Reactions were performed in individual wells of black 384-well low volume round bottom plates with the total reaction volume of 20 L. The standard reaction contained 20 mM Tris-HCl, pH 8.0, 10 mM KCl, 6 mM $MgCl_2$, 20 M ATP, 0.2 M self-primer RNA and 0.01 % Trition-X100 with 12 g/mL SARS-CoV-2 RdRp. After incubation of the SARS-CoV-2 RdRp with different concentrations of compound for 10 min at room temperature, the RNA and ATP were added then incubated for another 30 min at 30 °C. Then, the 15 L reaction system was added to 200 L RNA detection system and incubated for 5 min at room temperature, and the fluorescence was recorded immediately. The reaction with compound was set as Experience group; the reaction without compound was set as Control group; the reaction with only RNA and ATP was set as Blank group. The inhibition rate at each concentration point was calculated by the formula of (Control group - Experience group)/(Control group - Blank group).

1.4. FRET-base assay for SARS-CoV-2 3CL^{pro} activity

The inhibition of SARS-CoV-2 3CL^{pro} by GA171 was performed according to our previously described method [1]. In short, 120 nM of SARS-CoV-2 3CL^{pro} in the reaction buffer (0.1 M PBS, 1 mM EDTA, pH 7.4) incubated with indicated concentrations of GA171 or Vitamin K3 for 30 min at 37 °C and then 20 M fluorogenic substrate Dabcyl-KNSTLQSGLRKE-Edans (GenScript, Nanjing, China) was added to start the reaction. The fluorescence intensity was monitored continuously every 2 min for up to 20 min at 340 nm (excitation)/490 nm (emission) using Cytation 5 (BioTek, Winooski, USA). The IC_{50} value of GA171 was calculated by fitting the curve of normalized inhibition ratio with the test concentration.

1.5. Human ACE2 enzyme activity assay

The inhibitory activity of human ACE2 by GA171 was determined according to the previously published method [4]. For the inhibition assay of human ACE2, 100 nM ACE2 was incubated with indicated concentrations of GA171 or MLN-4760 (Beyotime, Shanghai, China) in reaction buffer (0.05 M 2-morpholinoethane-sulfonic acid, 0.3 M NaCl, and 10 M $ZnCl_2$, pH 6.8) for 30 min at 37 °C with 300 rpm continuous shaking. The fluorogenic substrate MCA-APK(Dnp)-OH (Beyotime, Shanghai, China) at a final concentration of 70 μM was added to initiate the reaction and the mixture was incubated at room temperature for 10 min. Fluorescence intensity was subsequently measured in black 96-well using microplate reader Cytation 5 (BioTek, Winooski, USA) with excitation wavelength set at 320 nm and emission wavelength set at 405 nm.

2. Results

2.1. GA171 inhibits 3CL and PL proteases but not RdRp and human ACE2

To further evaluate whether GA171 has other antiviral mechanisms besides blocking the SARS-CoV-2 S-RBD/ACE2 interaction, we determined the effect of GA171 on SARS-CoV-2 3CL^{pro}, PL^{pro}, RdRp, and human ACE2. The compounds Vitamin K3, GRL0617, Suramin, and MLN-4760 were used as the positive controls for SARS-CoV-2 3CL^{pro}, PL^{pro}, RdRp, and human ACE2, respectively [1, 5-7]. As shown in Fig.S7, GA171 exhibited inhibitory effects on 3CL^{pro} and PL^{pro} with IC_{50} values of 3.80 and 17.85 M, respectively. This suggests that GA171 dually targeting the recognition and replication stages of SARS-CoV-2. More importantly, our results demonstrated that GA171 has no effect on the catalytic function of human ACE2 at effective antiviral concentrations, emphasizing the specificity and safety of GA171 in inhibiting SARS-CoV-2.

Fig. S1. Validation of the positive compound Niclosamide. (A) The chemical structures of Niclosamide. (B) Inhibition rate of Niclosamide on NanoBiT, NanoLuc, and Cytotox. (C) Cytotoxicity of Niclosamide to hACE2/HEK293T cells. (D) Niclosamide blocked SARS-CoV-2-S pseudovirus from hACE2/HEK293T cells.

Fig. S2. RMSD analysis of predicted poses sampled MD simulations. (A) RMSD analysis. The bar plot shows the average RMSD values of MD-sampled binding poses with respect to their original docking poses. Only the heavy atoms of the salicylic acid warhead were involved in the RMSD calculations. The figure's bar and error bar denote the average RMSD and its corresponding standard deviation (SD) values, respectively. Two best-scoring binding poses in each potential binding pocket were carried out for MD simulations. Smaller RMSD and SD values indicate more stable poses, for example, poses 1 and 2 of site 1, and the greater values indicate unstable poses, such as poses 1 and 2 of site 2. Our basic rule for choosing potential pockets is as follows: 1) The RMSD and SD values of a pose should be comparatively low; for example, RMSD values less than 5 Å and SD less than 2 Å. 2) The pocket will be further considered even if one of two poses shows low RMSD and SD values. 3) GA171's warhead has direct polar interactions with its surrounding residues' side chains, primarily via salt-bridge interactions. (B) and (C) show docking poses in sites 3 and 4, respectively. Although site 3 and stie 4 have similar RMSD values and deviations, we finally chose site 3 for further experimental validation because its R346 interacts with GA171 with a potential salt-bridge interaction. By comparison, N343 in site 4 does not directly interact with GA171's warhead.

Fig. S3. Comparison of docking the ligand to the wild type and mutants. The left and right columns show docking information for the wild type and its corresponding mutants. The “blind” docking procedure was followed as described in the main text for the wild-type protein. The best-scoring poses and scores are provided for a potential binding pocket. For docking to the mutants, we mutated the corresponding residues and took the docked poses from the wild-type counterpart as the reference to define the docking pockets. The docking parameter “exhaustiveness” was set to 10.

Fig. S4. SPR binding curves (colored lines) obtained by passing different concentrations of SARS-CoV-2 S-RBD mutants over immobilized ACE2. (A) Binding affinity measurement of S-RBD (R346A) and ACE2 using SPR. (B) Binding affinity measurement of S-RBD (T430A) and ACE2 using SPR.

Fig. S5. Cytotoxicity of GA171 to BEAS-2B, Vero-E6 and MASMC cells. (A) GA171 exhibited low cytotoxicity to a variety of normal cells. (B) The CC_{50} values of GA171 in three types of cell lines.

Fig. S6. Docking GA171 to multiple potential binding proteins. SARS-CoV-2 RdRp, human ACE2, $3CL^{pro}$, and PL^{pro} were treated as the docking targets. The docking procedure was the same as that for S-RBD. The structure 7BV2 was used as the docking enzyme for RdRp [3]. The pre-catalysis state was constructed for the docking by removing the covalently incorporated Remdesivir. The ACE2 coordinates were extracted from the structure 6M0J [8]. $3CL^{pro}$ and PL^{pro} were extracted from the structures 6LU7 and 7JRN (chain A), respectively [9, 10]. The native-bound ligands and crystal structures were removed from the structures before docking. The best-scored docking poses are shown in yellow ball-and-stick fashion.

Fig. S7. The inhibitory activity of GA171 against SARS-CoV-2 $3CL^{pro}$, PL^{pro}, RdRp, and human ACE2. (A) GA171 showed inhibitory effect on SARS-CoV-2 $3CL^{pro}$ and PL^{pro}. Inhibition curves of GA171 against SARS-CoV-2 $3CL^{pro}$ (B) and PL^{pro} (C).

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