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Original Article

Corilagin prevents SARS-CoV-2 infection by targeting RBD-ACE2 binding

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ABSTRACT

Background: The outbreak of coronavirus (SARS-CoV-2) disease caused more than 100,000,000 people get infected and over 2,200,000 people being killed worldwide. However, the current developed vaccines or drugs may be not effective in preventing the pandemic of COVID-19 due to the mutations of coronavirus and the severe side effects of the newly developed vaccines. Chinese herbal medicines and their active components play important antiviral activities. Corilagin exhibited antiviral effect on human immunodeficiency virus (HIV), hepatitis C virus (HCV) and Epstein-Barr virus (EBV). However, whether it blocks the interaction between SARS-CoV-2 RBD and hACE2 has not been elucidated.

Purpose: To characterize an active compound, corilagin derived from Phyllanthus urinaria as potential SARS-CoV-2 entry inhibitors for its possible preventive application in daily anti-virus hygienic products.

Methods: Computational docking coupled with bio-layer interferometry, BLI were adopted to screen more than 1800 natural compounds for the identification of SARS-CoV-2 spike-RBD inhibitors. Corilagin was confirmed to have a strong binding affinity with SARS-CoV-2-RBD or human ACE2 (hACE2) protein by the BLI, ELISA and immunocytochemistry (ICC) assay. Furthermore, the inhibitory effect of viral infection of corilagin was assessed by in vitro pseudovirus system. Finally, the toxicity of corilagin was examined by using MTT assay and maximal tolerated dose (MTD) studies in C57BL/6 mice.

Results: Corilagin preferentially binds to a pocket that contains residues Cys 336 to Phe 374 of spike-RBD with a relatively low binding energy of -9.4 kcal/mol. BLI assay further confirmed that corilagin exhibits a relatively strong binding affinity with SARS-CoV-2-RBD and hACE2 protein. In addition, corilagin dose-dependently blocks SARS-CoV-2-RBD binding and abolishes the infectious property of RBD-pseudotyped lentivirus in HACE2 over-expressing HEK293 cells, which mimicked the entry of SARS-CoV-2 virus in human host cells. Finally, in vivo studies revealed that up to 300 mg/kg/day of corilagin was safe in C57BL/6 mice. Our findings suggest that corilagin could be a safe and potential antiviral agent against the COVID-19 acting through the blockade of the fusion of SARS-CoV-2 spike-RBD to hACE2 receptors.

Conclusion: Corilagin could be considered as a safe and environmental friendly anti-SARS-CoV-2 agent for its potential preventive application in daily anti-virus hygienic products.

Abbreviations: ACE2, angiotensin-converting enzyme 2; BLI, biolayer interferometry; COVID-19, coronavirus disease 2019; EBV, Epstein-Barr virus; EBV-DP, EBV DNA polymerase; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; HSV, herpes simplex; HIV, human immunodeficiency virus; IL-1β, interleukin-1β; MERS-CoV, Middle East respiratory syndrome coronavirus; NO, nitric Oxide; NS3, non-structural protein 3; PLpro, papain like protein; RBD, receptor-binding domain; RdRp, helicase and RNA dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; 3CLpro, 3-chymotrypsin like protein; S proteins, spike proteins; TNF-α, tumor necrosis factor α; WHO, World Health Organization.

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Introduction

The novel coronavirus pneumonia (COVID-19), which was caused by SARS-CoV-2 virus infection, has spread worldwide since 2019 and posed a huge threat to global health and economy. As of February 10, 2021, the COVID-19 has rapidly spread to 223 countries with 105,805,951 confirmed cases and 2,312,278 deaths all over the world according to the statistics of the World Health Organization (WHO). Although the median incubation period of SARS-CoV-2 virus was estimated as 7.76 days, around 90% cases were identified as 14.28 days. Up to now, with the nucleic acid test as the main adopted clinical diagnostic method, there was still no definite method to detect the early stage of SARS-CoV-2 infection. Since the virus is contagious during the incubation period, with an increasing trend of patients reported with an incubation period of longer than 14 days (Qin et al., 2020), therefore, undetected hidden infection cases remains a major problem for the disease control (Xiao et al., 2020; Mao et al., 2020). In addition, asymptomatic SARS-CoV-2 infected patients without typical symptoms such as cough, fever, dyspnea or physical fatigue, have also increased the risk of the disease transmission (Huff and Singh, 2020) in the community. Although different types of vaccines are developed against the SARS-CoV-2 in many countries, due to the limited time for the clinical trials, the side effects, safety and efficacy of the vaccines remained to be fully elucidated. Moreover, reported mutations of SARS-CoV-2 virus remain as the major challenge affecting the development and effectiveness of the vaccine. For instance, more contagious variants of SARS-CoV-2 with point mutations such as D614G, N501Y and E484K were identified with the abilities to induce immune escape (Plante et al., 2020; Wise, 2021).

Of note, the spike proteins of SARS-CoV-2 virus are adopted as the antigens of vaccines, as well as the targets of the antibody cocktail therapies, therefore, continuous monitoring on the efficacy of the current developed vaccines is highly required.

SARS-CoV-2 virus is an enveloped and single stranded RNA virus which belongs to the subgenus sarbecovirus or thocoronavirinae family (Qu et al., 2020). SARS-CoV-2 virus contains genome encoding the replicas including 3-chymotrypsin like protein (3CLpro), papain like protein (PLpro), helicase and RNA dependent RNA polymerase (RdRp), which are the key enzymes for the replication of the viruses (Swift and People, 2020). Other genome mainly encodes structural proteins, including spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins. S protein and M protein are mainly involved in the assembly process of virus, and N protein encapsulates genomic viral RNA to form nucleoprotein complex (Papageorgiou and Mohsin, 2020). Among them, spike protein recognizes the host cell receptor and mediates membrane fusion, which is crucial for virus particles to enter and infect the host cells. Similar to SARS virus in 2003, SARS-CoV-2 enters the cells by recognizing ACE2 protein of human host (Swift and People, 2020). Recent research reported that the spike protein of the SARS-CoV-2 virus is randomly distributed on the virus surface, which enhances the positional variability and flexibility for virus to bind to the ACE2 receptor for higher viral infectivity (Yao et al., 2020). Receptor binding domain (RBD) of spike protein is directly involved in the recognition of host receptor (Lan et al., 2020), and alternating the amino acid in this region can lead to the change of species tropism and infection characteristics of SARS-CoV-2 (Plante et al., 2020). Therefore, the identification of specific antibody or novel small-molecule inhibitor targeting the spike protein is also an important approach for the prevention and drug design of COVID-19.

Chinese herbal medicines and their active components play important antiviral activities. Yeo et al. reported that Phyllanthus urinaria L. (Phyllanthaceae) extract, which contains corilagin as a major component, exhibited antiviral effect on human enterovirus 71 (EV71) and coxsackievirus A16 (CA16) in vitro (Yeo et al., 2015). Another studies reported that Phyllanthus amarus Schumach. & Thonn. (Phyllanthaceae) containing corilagin and geraniin demonstrated inhibitory effects on human immunodeficiency virus (HIV) in in vitro and in vivo model (Notka et al., 2004). Other reports found that corilagin, excoecariphenol D, ganerian, and chebulagic acid derived from Excoecaria agallocha L. (Euphobiaceae) showed potential inhibition of hepatitis C virus (HCV) NS3-4A protease (Xiao et al., 2012). Interestingly, corilagin, a kind of gallotannin, can be found in many natural plants such as Dimocarpus longan Lour. ( Sapindaceae) (Li et al., 2018a), Phyllanthus emblica L. (Phyllanthaceae) (Jantan et al., 2019), this compound has been reported to show a board inhibitory effect on Epstein-Barr virus (EBV), hepatitis C virus (HCV), herpes simplex (HSV), human immunodeficiency virus (HIV) and other viruses. For instance, it was reported to inhibit the activity of EBV DNA polymerase (EBV-DP) and reduce the ability of EB viral replication (Liu et al., 1999). Similarly, it also inhibited the activity of non-structural protein 3 (NS3) serine protease, a key protein for HCV replication (Reddy et al., 2018). On the other hand, corilagin has demonstrated to suppresses the inflammatory effect of HSV on the mouse brain via reducing the release of inflammatory factors such as TNF-α, IL-1β and NO (Guo et al., 2010). It also blocked HIV-1 adsorption by inhibiting HIV-1 integrase, reverse transcriptase and protease activities (Notka et al., 2004). However, its antiviral effect towards SARS-CoV-2 remains unclear. In the current study, corilagin has been identified for the first time as a small-molecule inhibitor for targeting the RBD of spike protein in SARS-CoV-2. Our results showed that corilagin blocks the spike-RBD binding on hACE2 receptor and abolishes the infectious property of RBD-pseudotyped lentivirus in hACE2 overexpressing HEK293 cells, which mimicked the viral infection of SARS-CoV-2 in human cells. Notably, SARS-CoV-2 has similar adsorption spike proteins to HIV (Hung et al., 2020), further suggesting that corilagin has the potential to inhibit the adsorption and infection of new coronaviruses on human host cells, and making it as a potential drug candidate against COVID-19.

Material and methods

Chemical and reagents

Corilagin (purity > 99%) was purchased from Chengguang Biological Technology Co., Ltd (Baoji, China) and was also verified by NMR and UPLC-UV (Fig. S1-3). Its chemical structure was shown in the Fig. 1. DMSO was supported by Sigma-Aldrich (St. Louis, MO, USA). MT3 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazoliumromide) powder was obtained from Thermo Fisher (Waltham, MA, USA). FluorSave reagent was purchased from Calbiochem (San Diego, CA, USA). The ACE2: SARS-CoV-2 Spike Inhibitor Screening Assay Kit (Cat: #79936) was purchased from BPS Biosciences (San Diego, CA, USA). Purified ACE2-His tag protein and SARS-CoV-2 RBD protein were supported by Sino
Preparation of the target protein and ligand

The information of the RBD/ACE2-B0AT1 complex of SARS-CoV-2 was downloaded from the protein data bank (ID 6M17). The target was prepared by using UCSF Chimera. In this study, the receptor binding domain (RBD) amino acid chains representing the sodium-dependent neutral amino acid transporter B (0) AT1 was used, whereas the angiotensin-converting enzyme 2 and the second receptor binding domain were removed accordingly. In addition, all non-standard residues such as water, N-acetyl glucosamine and zinc were also removed. The PDB file was then uploaded and processed by using Flare (Cresset, version 3.0) software. Hydrogens were added and optimal ionization states were assigned for each residue. To maximize hydrogen bond interactions and minimize steric strain, the spatial positions of polar hydrogens were optimized. His, Asn and Gln residue side chains were also optimized for their orientation, whereas the unresolved side chains were reconstructed. Corilagin was downloaded from Pubchem (CID 73568) in SDF format. Energy minimization and conversion to mol2 file format was performed by using OpenBabel software.

Molecular docking

To predict the preferred binding pocket of corilagin on the RBD, blind docking prediction was conducted by using SwissDock docking program which can simulate all possible binding modes for the ligand. The most preferred binding modes were constructed and clustered at a specified binding pocket. All clusters were visualized with UCSF Chemiera. A cluster is a predicted binding pocket on the target protein and each cluster was considered as the interacting amino acid. To obtain the best pose at the predicted binding region, site specific docking was conducted by using Flare (Cresset, version 3.0) software. SDF file format of the small-molecule, corilagin, was uploaded and processed by using default settings. The grid was selected to include the predicted binding pocket identified from the SwissDock results. The best binding pose for corilagin was selected for further analysis.

Molecular dynamics simulation

The stability of the interaction of corilagin with the SARS-CoV2 RBD was further evaluated. The best binding pose of corilagin with SARS-CoV2 RBD obtained from the molecular docking prediction was subjected to MD simulation using GROMACS package version 2020.3. The topology of SARS-CoV2 RBD protein was constructed using the CAHRRM36 force field. The selected binding pose of corilagin was converted to Mol2 file format using Avogadro software. The topology of corilagin was prepared from the CgEnff server. Protein-ligand complexes were generated and then solvated in a dodecahedral water box using an explicit SPC water model. The system was neutralized by adding appropriated counter ions. To minimize the energy, the system was allowed to converge at the tolerance of 1000 kJ mol⁻¹ nm⁻¹ with 500 steps of steepest descent. The system was then equilibrated in two phases. The first phase was implemented for NVT equilibration at 300 K. The second phase was performed for NPT equilibration at 1 bar pressure. The MD simulation was executed to 10 nano seconds (picosec). Root mean-squared deviation (RMSD) and interaction energy were stored in the trajectory for every 1 picosecond (picosec) and were analyzed by using Grace Software.

Bilayer interferometry (BLI) assay

ACE2-His tag protein (Sino Biological) was immobilized onto the Ni-NTA probes purchased from Fortebio (Fremont, CA, USA). Purified SARS-CoV-2 RBD peptide (Sino Biological) were conjugated with biotin using EZ-Link™ Sulfo-NHS-Biotin (Genemore, Shanghai, China) according to the manufacturer’s protocol. Then, the biotinylated SARS-CoV-2 RBD peptide were immobilized onto Super Streptavidin (SSA) biosensors (Fortebio). SARS-CoV-2 RBD peptide (Sino Biological) were diluted into different concentrations ranging from 0.625 μg/ml to 10 μg/ml. After the setup of the baseline with PBS containing 2% DMSO (Sigma-Aldrich), biosensor tips were immersed into the wells containing serial dilutions of SARS-CoV-2 RBD peptide for 300 seconds (sec) of association, followed by a dissociation step of 300 sec. The KD value was calculated using a 1:1 binding model in Data Analysis Software 9.0 (Fortebio). Corilagin was serially diluted from 100 μM to 3.13 μM with PBS.

Enzyme-linked immunosorbent assay (ELISA)

The ACE2: SARS-CoV-2 Spike Inhibitor Screening Assay Kit (Cat: #79936) was purchased from BPS Biosciences. Nickel-coated 96-well white microplates were coated with 1 μg/ml ACE2-His-tag protein by following the manufacturer’s protocol. Briefly, 1 ng/μl SARS-CoV-2 RBD-Fc-tagged protein was added to ACE2-His-tag coated test wells in the presence of 0, 25, 50 or 100 μM of corilagin. Wells without corilagin and SARS-CoV-2 RBD protein were set as blank control. Finally, the plate was treated with Anti-mouse-Fc-HRP followed by addition of ELISA ECL substrate solution containing anti-horseradish peroxidase into the microplate wells followed by the chemiluminescence measurement using the luminometer (Tecan, Männedorf, Switzerland). Quantification bar charts represented the ELISA results from 3 independent experiments with the mean ± S.D.

MTT assay

Cell viability (IC₅₀) was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Corilagin was dissolved in DMSO as stock concentration at 100 mM and stored at −20 °C before use. Beas-2B, LO2 and HEK293 cells were seeded in 96-well plates (6000 cells/well) respectively. Cells were then treated with different concentrations (0-100 μM) of corilagin for 72 h. After treatment, 10 μl of MTT solution (5 mg/ml) was added into each well for a further incubation of 4 h. Subsequently, 100 μl of 10% SDS buffer supplemented with 0.01 M HCl were added to each well for overnight incubation. Absorbance optical density (A) at 570 nm was measured by the plate reader (Tecan, Männedorf, Switzerland). The percentage of cell viability (%) was calculated by the formula (A /[A - A ] × 100).

Immunocytochemistry (ICC) assay

HEK293 cells transfected with ACE2-EGFP (Vectorbuilder) were seeded overnight at a density of 5000 cells/well on a sterile coverslip inside a 24-well plate. To begin, corilagin and SARS-CoV-2-RBD-mFc protein (Sino Biological) were co-incubated for 30 min before being
added into the cells for a further incubation of 40 mins. After treatments, cells were fixed with 4% PFA for 10 min and blocked with 3% BSA for another 30 min. Cells were then incubated with anti-mouse IgG Fc TRITC antibody purchased from Invitrogen (Carlsbad, CA, USA) for 2 h. The coverslips with cells were mounted with FluorSave reagent (Calbiochem) and visualized by confocal microscope with Leica SP8 (Wetzlar, Germany). For semi-quantitative determination, fluorescence images were analysed by the Image J. The optical density of the TRITC-labeled SARS-CoV-2-RBD was measured. The reduction (%) of TRITC optical density compared to the un-treated control wells were calculated as the neutralizing potency. Quantification bar chart represented the data from 3 independent experiments.

S-pseudovirus-based viral infection assay

HEK293 cells were transfected with human ACE2-mCherry construct (Vectorbuilder) for 24 h. After that, 1 x 10^5 transfected cells were seeded overnight on a coverslip inside a 24-well plate. Corilagin, RBD-pseudotyped lentivirus (Vectorbuilder) and polybrene (Vectorbuilder) were premixed in medium before being added into the cells. After 12 h, the cells were replaced with normal medium and incubated for a further 48 h. The cells were then washed with PBS and fixed with 4% PFA. The coverslips with cells were mounted with FluorSave reagent (Calbiochem). Cells were visualized by Leica SP8 confocal microscope and then subjected to semi-quantitative determination as described before. In addition, a standard calibration curve on viral infection using 46.6, 37.3, 23.3, 4.66, 0 x 10^6 TU of RBD-pseudotyped lentivirus were established for the evaluation of virus inhibition. Fluorescence intensity of cells for every titer of viral infection were collected and then calculated by Image J. Quantification bar chart represented the data from 3 independent experiments.

Viral infection formula: y = 286200x - 98383, R^2 = 0.9729

Maximum-tolerate dosage (MTD) study in C57BL/6 mice

Male C57BL/6 mice at the age of 6–8 weeks were obtained from SPF Biotechnology Co., Ltd (Beijing, China). All experiments were carried out in accordance with the “Institutional Animal Care and User Committee Guidelines” of the Macau University of Science and Technology. C57BL/6 mice were randomly divided into 3 groups to determine the maximum-tolerate dosage of corilagin. With the control mice group treated with same volume of sterilized water, treatment mice groups were orally administered with either low dose of corilagin (200 mg/kg, n = 10) or high dose of corilagin (300 mg/kg, n = 10) for 7 consecutive days. The health condition of the C57BL/6 mice were monitored and evaluated based on their changes in body and vital organ weight. Vital organs including liver, spleen and kidney were collected after scarification.

Statistical analysis

All experiments were repeated for 3 times. Results are shown as mean ± S.D. All statistical analysis was examined by using Graphpad Prism software 8 (San Diego, CA, USA). Statistical significance among multiple group comparisons were performed by one-way analysis of variance (ANOVA). P value < 0.05 was considered as statistically significant.

Results

Corilagin binds and interacts with Spike-RBD domain of SARS-CoV-2 and ACE2 receptor

In this study, we investigated the effect of corilagin on SARS-CoV-2 by using the docking software SwissDock that can predict the preferential binding site of corilagin. The binding pocket with the highest number of clusters was specified as the preferential binding site (Fig. 2A). As shown in Fig. 2B, the preferential binding pocket for corilagin involved residues Cys 336 to Phe 374. To specify the best binding position, site specific docking was conducted by using Flare (Cresset) on slow accurate mode. The grid was adjusted to include the predicted binding pocket. The dG, VS and LG scores were recorded for the best binding pose as shown in Fig. 2A. The best binding pose was considered for further analysis by molecular dynamics simulation. In molecular dynamics simulations, the root mean square deviation (RMSD) is the standard measure of atomic distances between trajectories. It is a measure of how much a system has changed over time. When the RMSD plot with time reaches a plateau, this indicates that the system has reached stability. Fig. 2C demonstrated the RMSD fluctuation along with the molecular dynamics (MD) simulation time. The time for considerable structural change is noticed within 4 nanosec. The average interaction energies were calculated throughout the simulation from the electrostatic and Lennard-Jones interaction energies. Electrostatic interaction energy was found to be -136.6 ± 12 kJ/mol and the Lennard-Jones interaction energy was found to be -23.9 ± 8.4 kJ/mol. Taken together, computational docking and molecular dynamics simulations suggest that corilagin may bind and interact appropriately with the Spike-RBD of SARS-CoV-2.

According to our previous data, we confirmed that SARS-CoV-2 RBD targeted and bound to hACE2 in a dose-dependent manner (Chen et al., 2021). To further validate the binding affinity of corilagin towards the Spike-RBD protein, which could ultimately affect the interaction between SARS-CoV-2 RBD and hACE2, bio-layer interferometry (BLI) analysis was conducted. Firstly, the Spike-RBD peptide was immobilized onto the biosensor coated with super streptavidin (SSA) via biotinylation for the measurement on the binding affinity of corilagin with the labelled probe. Through the immobilization of the biotinylated Spike-RBD onto the biosensor surface and its subsequent exposure to different concentrations of corilagin, the association and dissociation curves were monitored in a real-time mode. As shown in Fig. 3A, corilagin was found to dose-dependently bind to Spike-RBD, whereas the binding curves of RBD suggested that a simple 1:1 binding mode occurred with R^2 = 0.9219. Consistent with the computational docking data, the equilibrium dissociation constant (KD) of the interaction between RBD and corilagin was 17.4 μM (Steady state analysis, lower panel). Of note, ACE2 has been reported to be a functional receptor for SARS-CoV-2 to enter the host target cells (Bourgonje et al., 2020), therefore small-molecules adhesion on ACE2 may also affect the attachment and viral infection of SARS-CoV-2. His-tagged ACE2 immobilized onto the biosensor coated with nickel-nitrioltriacetic acid (Ni-NTA) was utilized to measure the binding affinity with corilagin. Interestingly, corilagin was confirmed to dose-dependently bind to ACE2, whereas the binding curves suggested that corilagin bind to ACE2 in a simple 1:1 binding mode with R^2 = 0.8361 (Fig. 3B). The KD of the interaction between ACE2 and corilagin was 1.39 μM (Steady state analysis, lower panel), suggesting that corilagin may bind and interact with both viral Spike-RBD and host cells ACE2 receptor for anti-viral infection.
Fig. 2. Computational docking prediction of corilagin. A) Molecular docking result showed the best binding pose of interaction, and B) the residues involved and types of interaction of corilagin with the receptor binding domain of the SARS-CoV-2 spike protein. C) Root mean square deviation (RMSD) plot of the SARS-CoV-2 spike protein receptor binding domain (RBD) backbone alone and in complex with corilagin during the 10ns molecular dynamics simulation.
Corilagin interferes with the binding between Spike-RBD peptide and ACE2 receptor

To further determine whether corilagin can inhibit the interaction of Spike-RBD-ACE2, the ELISA assay coated with ACE2-His-tag protein on the 96-well white microplates was adopted for the competition study. The percentage of inhibition as reflected by the differences in the optical density of cells with or without the corilagin treatment were used to determine the half maximal inhibitory concentration (IC₅₀). Results showed that corilagin dose-dependently blocked the binding of Spike-RBD peptide to ACE2 at an IC₅₀ of 24.9 μM (Fig. 4), demonstrating the potential inhibitory effect of corilagin on the fusion between the viral Spike-RBD and ACE2 receptor of the host cells.

Corilagin abolishes the binding and infection of recombinant RBD pseudovirus in ACE2 overexpressing human cells

To visualize the in vitro antiviral effect of corilagin on inhibiting Spike-RBD and ACE2 binding, SARS-CoV-2 Spike-RBD protein-based ICC assay and S-pseudolentivirus infection assay were conducted. As shown in Fig. 5A, SARS-CoV-2 Spike-RBD-mFc protein (TRITC) and hACE2 transfected cells (EGFP) were co-localized in the untreated
revealed that corilagin inhibited S-pseudolentivirus infection in a dose-dependent manner. Consistently, hACE2 HEK293 cells were pre-treated with 50 μM corilagin, hACE2 HEK293 cells were completely infected by S-pseudolentivirus (Fig. 6A). As reflected by a real-time BLI assay, HEK293 cells overexpressed with hACE2 (mCherry) were infected with Spike-RBD-hACE2. Furthermore, RBD-pseudotyped lentivirus infection assay was conducted to evaluate the efficacy of corilagin in antiviral infection. Consistent with the SARS-CoV-2 Spike-RBD protein-based 1CC assay, HEK293 cells overexpressed with hACE2 (mCherry) were completely infected by S-pseudolentivirus (Fig. 6B). As reflected by a weak EGFP fluorescence signal, the viral infection study further revealed that corilagin inhibited S-pseudolentivirus infection in a dose-dependent manner (Fig. 6B). To further investigate the protective effect of corilagin, hACE2 HEK293 cells were pre-treated with 50 μM corilagin for 24 h. The viral inhibition of 25 μM corilagin treatment in pre-treated group, has upregulated for 12.5% compared to cells without corilagin pre-treated (Fig. S4), suggesting pre-treated cells with corilagin also has a protective effect. In conclusion, corilagin may be considered as a potent candidate for SARS-CoV-2 virus entry inhibitor, which can protect human cells from viral infection.

Toxicology study of corilagin in C57BL/6 mice

The toxicity of corilagin was evaluated both in vitro and in vivo by using 3 different human normal cell lines including lung epithelial cells (Beas-2B), normal liver hepatocytes (LO2) and human embryonic kidney 293 cells (HEK293), as well as C57BL/6 mice. As shown in Fig. 7A, corilagin exhibited no cytotoxicity to the selected human normal cells at a maximum concentration of 100 μM. To further investigate the safety of corilagin in vivo, maximum-tolerable dosage (MTD) was conducted by using C57BL/6 mice model. Apparently, C57 mice orally administrated with 200 mg/kg/day or 300 mg/kg/day of corilagin showed no toxic or harmful effect to animals as revealed by a survival rate of 100%. Besides, drop in body and vital organs weight were not observed after a 7-days treatment course of corilagin (Fig. 7B).

Discussion

The life-threatening consequences of the new coronavirus pandemic remain severe due to scarcity of selective targeted therapies and vaccination strategies. Hence, it is imperative to investigate and develop alternatives such as antiviral phytochemicals to inhibit SARS-CoV-2 infection. Corilagin existed in several plants such as *Alchornea glandulosa* Poep. (Euphorbiaceae) (Rangkadiok et al., 2005), *Caesalpinia coriaria* (Jacq.) Willd. (Fabaceae) (Darwish et al., 2016) and *Punica granatum* L. (Lythraceae) (Ngotka et al., 2003) has aroused scientific attention because of its anti-tumor (Li et al., 2018b), hepatoprotective (Tang et al., 2019), and anti-inflammatory (Kolodziej et al., 2005) activities. Notably, corilagin has been reported to inhibit HCV replication by suppressing NS3 protease enzyme activity at sub-micromolar concentrations (Yue et al., 2005). Furthermore, corilagin effectively attenuated HSV-1-induced encephalitis (Tong et al., 2016) and inhibited HIV both in vitro and in vivo (Xu et al., 2000). Although recent computational docking analysis has depicted the possible interaction of corilagin with one of the binding sites of SARS-CoV-2 main protease (CoV-2 Mpro) (Santos-Filho, 2020), both in vitro and in vivo study of corilagin on SARS-CoV-2 are lacking. In addition to computational docking, BLI analysis which is a real-time detection method for bio-molecular interactions, were adopted to confirm the effect of corilagin in interacting with the SARS-CoV-2 RBD protein. Structure-based drug design has become a valuable and indispensable tool in drug discovery. It includes various methods which utilize the three-dimensional structural information gathered from biological targets for studying their interaction with small molecules. Computational docking and molecular dynamics are among the most frequently used methods to study how small-molecules recognize and interact with the target proteins. In the present study, both computational docking and molecular dynamics were adopted to study the molecular interactions between corilagin and the RBD of the COVID-19 S-protein. We demonstrated that corilagin preferentially bound to the pocket that involved residues Cys 336, Phe 338, Gly 339, Phe 342, Val 362, Ala 363, Asp 364, Val 367, Leu 368. The results of the site-specific docking showed that the best binding pose of corilagin interacts with a relatively low binding energy of -9.4 kcal/mol. It was found from the 10 nanosec molecular dynamics simulation that the selected binding pose was stable for the last 6 nanosec. The binding interaction depended more on electrostatic interaction compared to van der Waal’s interactions. This could be explained by the 5 hydrogen bonds shown in Fig. 2. A recent study investigated the cryo-electron microscopy structure of SARS-CoV-2 spike (S) glycoprotein revealed that the RBD tightly bound to linoleic acid in 3 composite binding pockets. Interestingly, our predicted binding pocket was found to be one of these binding pockets. In fact, a similar pocket was found in several highly pathogenic coronavirus including the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). It has shown that binding of linoleic acid to this pocket could stabilize the S-protein in the closed conformation and inhibit its transformation into the open conformation necessary for its binding to its receptor angiotensin-converting enzyme 2. Therefore, it is postulated that corilagin may irreversibly lock the S-protein in the closed conformation and interfere its interaction with the receptor. Based on the interactions between corilagin and the SARS-CoV-2-RBD revealed by molecular docking analysis, we confirmed that corilagin possesses binding affinity towards the RBD protein. Consistently, real-time BLI assay confirmed that corilagin possess binding affinity to both SARS-CoV-2-RBD and hACE2 receptor with a Kd of 17.4 μM and 1.39 μM, respectively. ELISA assay further confirmed that corilagin inhibited the binding of SARS-CoV-2-RBD to hACE2 receptor in
a dose dependent manner. In addition, the inhibitory effect of corilagin on the binding of RBD to hACE2 was further visualized by immunocytochemistry analysis. While corilagin inhibited RBD binding to cell membrane of HEK293, most importantly, pre-incubation of corilagin with RBD-pseudotyped lentivirus also abolished the infectious property of virus in hACE2 overexpressing HEK293 cells, which mimicked the entry of SARS-CoV-2 virus in human host cells. Finally, cell viability assay showed that treatment with corilagin (100 µM) did not induce cytotoxicity to several human normal cell lines (Beas-2B, LO2 and HEK293), which is consistent with the reported data that up to a maximum concentration of 1000 µM, corilagin did not show any toxicity in Huh7 cells (Reddy et al., 2018). More importantly, we observed that there was no drop in body and pivotal organs weight in C57BL/6 mice, suggesting that corilagin is relatively safe for both external and internal use. Of note, corilagin is reported to be a potential inhibitor of SARS-CoV-2 targeting viral RNA-dependent RNA polymerase (Li et al., 2020).

Fig. 5. Corilagin suppresses the binding of Spike-RBD on ACE2 receptor in HEK293 cells. A) HEK293 cells were transfected with hACE2-EGFP (green). The transfected HEK293 cells were incubated with mFc-tagged SARS-CoV-2-RBD protein with or without corilagin (25–100 µM). Mouse IgG Fc TRITC antibody (red) was used to visualize the binding of SARS-CoV-2-RBD proteins on cell surface. All images were captured by confocal microscopy using a Leica SP8 (× 40 oil immersion objective lens). B) Images of Spike-RBD-ACE2 binding intensity were quantified by Image J. Data were expressed as mean ± S.D., n = 3; * p < 0.05, one-way ANOVA analysis.
Together with our findings, corilagin may have double targets on SARS-CoV-2 for prevention of viral entry and replication.

**Conclusion**

Our results indicated that natural corilagin without cytotoxicity and toxicity to normal cells and C57BL/C mice could be considered as a safe and environmental friendly anti-viral agent for different purposes, for example, as the ingredients of anti-viral air spray, detergents or disinfectants to fight against the COVID-19 via inhibiting the fusion of spike-RBD of SARS-CoV-2 to cellular ACE2 receptors.

**CRediT authorship contribution statement**

Li Jun Yang: Methodology, Writing – original draft, Data curtion, Formal analysis. Rui Hong Chen: Methodology, Writing – original draft, Data curtion, Formal analysis. Sami Hamdoun: Data curtion, Formal analysis, Methodology. Paolo Coghi: Methodology,
Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

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