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GMI, a protein from *Ganoderma microsporum*, induces ACE2 degradation to alleviate infection of SARS-CoV-2 Spike-pseudotyped virus

Hsin Yeh a,1, Di Ngoc Kha Vo b,1, Zhi-Hu Lin a, Ha Phan Thanh Ho b, Wei-Jyun Hua a,c, Wei-Lun Qiu a, Ming-Han Tsai b,c,d,e, Tung-Yi Lin a,c,d,e,**

a Institute of Traditional Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
b Institute of Microbiology and Immunology, National Yang Ming Chiao Tung University, Taipei, Taiwan
c Program in Molecular Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
d Research Center for Epidemic Prevention, National Yang Ming Chiao Tung University, Taipei, Taiwan
e Biomedical Industry Ph.D. Program, National Yang Ming Chiao Tung University, Taipei, Taiwan

** Corresponding author at: Institute of Microbiology and Immunology, National Yang Ming Chiao Tung University, 155 Li-Nung St., Sec. 2, Shipai, Beitou, Taipei 112, Taiwan.

* Corresponding author at: Institute of Traditional Medicine, National Yang Ming Chiao Tung University, 155 Li-Nung St., Sec. 2, Shipai, Beitou, Taipei 112, Taiwan.

E-mail addresses: vnkhadil@nycu.edu.tw (D.N.K. Vo), hptha.ls09@nycu.edu.tw (H.P.T. Ho), karta603@gm.ym.edu.tw (W.-J. Hua), m.tsai@nycu.edu.tw (M.-H. Tsai), biotuny@gmail.com, tylin99@nycu.edu.tw (T.-Y. Lin).

1 These authors equally contribute to this study.

**Abstract**

*Background:* Severe Acute Respiratory Syndrome Coronavirus Type 2 (SARS-CoV-2) induces a global serious pandemic and is responsible for over 4 million human deaths. Currently, although various vaccines have been developed, humans can still get SARS-CoV-2 infection after being vaccinated. Therefore, the blocking of SARS-CoV-2 infection may be potential therapeutic strategies. *Ganoderma microsporum* immunomodulatory protein (GMI), a small fungal protein, is cloned from *Ganoderma microsporum*. It exhibits anti-cancer and immunomodulatory functions. Currently, it is still unclear whether GMI involves in interfering with viral infection.

*Purpose:* This study aimed to examine the potential functions and mechanisms of GMI on inhibiting SARS-CoV-2 pseudovirus infection.

*Methods:* The effects of GMI were examined in vitro on ACE2 overexpressing HEK293T (HEK293T/ACE2) cells exposed to SARS-CoV-2 Spike lentiviral pseudovirus encoding a green fluorescent protein (GFP) gene. The infection efficacy was determined using fluorescence microscopy and flow cytometry. The protein level of ACE2 was verified by Western blot. The effects of GMI on cell viability of HEK293T/ACE2 and lung epithelial WI38–2RA cells were determined by MTT assay. Mice received GMI via nebulizer.

*Results:* GMI did not affect the cell viability of HEK293T/ACE2, WI38–2RA and macrophages. Functional studies showed that GMI inhibited GFP expressing SARS-CoV-2 pseudovirus from infecting HEK293T/ACE2 cells. GMI slightly interfered the interaction between ACE2 and Spike protein. GMI interacted with S2 domain of Spike protein. Specifically, GMI dramatically reduced ACE2 expression in HEK293T/ACE2 and WI38–2RA cells. Mechanistically, GMI induced ACE2 degradation via activating protein degradation system, including proteasome and lysosome. Abolishing proteasome and lysosome by MG132 and bafilomycin A1, respectively, rescued GMI-reduced ACE2 levels. In addition, GMI triggered dynamin and lipid raft-mediated ACE2 endocytosis. ACE2 levels were downregulated in the lung tissue after the mice inhaling GMI.

*Conclusions:* GMI prevents SARS-CoV-2 pseudovirus infection via induction of ACE2 degradation in host cells. Our findings suggest that GMI will be a potential prevention agent to alleviate SARS-CoV-2 infection.

**Abbreviations:** ACE2, angiotensin-converting enzyme 2; BafA1, bafilomycin A1; CHX, cycloheximide; Exp, experiment; FBS, fetal bovine serum; FIPs, fungal immunomodulatory proteins; GFP, green fluorescent protein; GMI, Ganoderma microsporum immunomodulatory protein; HEK293T/ACE2, ACE2 overexpressing HEK293T cells; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; MjCD, methyl-β-cyclodextrin; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2; SARS-CoV-2-S, SARS-CoV-2 Spike; WI38–2RA, normal human lung epithelial WI-38 VA-13 subline 2RA.
Introduction

Coronavirus disease-19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still a global pandemic. Currently, there are already vaccines that can be used to prevent this viral infection; however, the rate of vaccine delivery is not universal in many countries. In addition, some reports show that SARS-CoV-2 may break through the vaccine protection and still cause risk of infection (Abbasi, 2021; Kustin et al., 2021). Therefore, in addition to the development of vaccines, the use of other strategies to improve vaccine efficiency or reduce viral infections is an urgent and important concern.

It is well-known that Spike (S) protein of SARS-CoV-2 is a major protein which specifically recognizes and binds to the angiotensin converting enzyme 2 (ACE2) of host cells, resulting in infection (Lan et al., 2020). Therefore, it has been an increase in the number of studies that focus on how to block SARS-CoV-2-S/ACE2 interactions, which could become a potential therapeutic direction to prevent infection. Currently, recombinant ACE2 proteins, ACE2-neutralizing antibodies, engineered ACE2 traps, and others have been investigated to exploit their potential efficacy of interfering with SARS-CoV-2-S/ACE2 interactions (Glasgow et al., 2020; Zamorano Cuervo and Grandvaux, 2020; Zoufaly et al., 2020). Interestingly, in addition to focusing on the development of blocking the interaction between ACE2 and Spike, one study has pointed out that a small molecule, lenalidomide, can be used to induce ACE2 degradation, which can effectively alleviate viral infections (Su et al., 2021). This study provides a new concept of the SARS-CoV-2 infection reduction via the induction of ACE2 degradation in host cells.

Ganoderma microsporum immunomodulatory protein (GMI; Fig. 1) is a kind of fungal immunomodulatory protein (FIP) from Ganoderma microsporum. In the past ten years, GMI has been proven to have multi-faced anti-cancer activities, especially on lung cancer. For examples, GMI inhibits EGFR-mediated cell motility of lung cancer cells (Lin et al., 2010). Meanwhile, GMI induces autophagic cell death of various lung cancer cells in vitro and in vivo via p53 and mTOR pathways (Hsin et al., 2011; 2012; 2015). Moreover, increasing evidence shows that GMI triggers protein degradation pathways, which result in the inhibition of lung cancer cells survival. Hsin et al. demonstrated that GMI triggers apoptosis in lung cancer cells via induction of proteasome-dependent β-catenin degradation (Hsin et al., 2018). GMI inhibits cell viability of pemetrexed-resistant lung cancer cells via induction of autophagic CD133 degradation (Hsin et al., 2020). These findings suggest that GMI may target lung cells and induce protein degradation pathways.

In this study, we conducted a SARS-CoV-2 Spike protein conditioned pseudovirus and ACE2 overexpressing HEK-293T (HEK293T/ACE2) cells to analyze the effects of GMI on virus infection. We dissected the potential mechanisms of GMI on virus infection. Specifically, we focused on the interaction between GMI and SARS-CoV-2 Spike protein and therefore further investigated whether GMI could modulate ACE2 expression on HEK293T/ACE2 and lung WI38–2RA cells. We also examined the role of GMI on ACE2 stability.

Preparation of GMI

GMI (Cat: 767593; Batch# MM-13AI28TU), dissolved in PBS, was obtained from MycoMagic Biotechnology Co., Ltd. (New Taipei, Taiwan). The details of construction, expression and purification of GMI has been described elsewhere (Lin et al., 2010). Briefly, GMI is a recombinant protein cloned from G. microsporum by PCR using forward primer 5′-CGTTCGACTACCTCGAACTGGG-3′ and reverse primer 5′-GTTCCACTGCGGCGATGA-3′ with extra base for the restriction enzyme cutting sites, EcoRI/XbaI, respectively, for the expression vector construction in pPICZ (Invitrogen, Carlsbad, CA, USA), transformed into Pichia pastoris KM71H (Invitrogen) and then handled per the manufacturer’s instruction. The P. pastoris transformant was cultured in buffered complex medium supplemented with 1% (v/v) glycerol as a carbon source. As the culture reaches log phase growth, cells were subsequently incubated and amplified into buffered complex medium with 0.5% methanol every 24 h for 48 h for the GMI secreted expression. The fermentation filtrated containing GMI was then purified by a one-step chromatography protocol, Ni-NTA column (Cat# 17-5318–02, Cytiva, Washington, Wash, U.S), and the target GMI was eluted by a gradient of 100–250 mM imidazole at pH 7.4. Finally, the GMI was dialyzed with PBS for the long-term storage and release-testing. The sample containing GMI was separated by SDS-PAGE under reducing condition and stained with Coomassie Brilliant Blue R-250; Endotoxin levels of GMI were performed by Limulus amebocyte lysate assays (<0.10 EU/µg) (The product datasheet and certificate of analysis was included in the supplemental information). The structure and amino acid sequence of GMI (PubChem SID: 461501955, PDB ID: 3KCW) were shown in Fig. 1.

Cell culture

HEK293T cell line is a derivative of HEK293 cells that contains the SV40 T-antigen (ATCC: CRL-3216). HEK293T cells with stable expression of human ACE2 (HEK293T/ACE2) were obtained after transduction with lentiviruses carrying hACE2 and blasticidin resistance genes. HEK293T/ACE2 cells were selected with 10 µg/ml blasticidin (Cat# A29168–070; Gibco). All cells were cultured in RPMI-1640 medium (Cat# 31800–022; Gibco) supplemented with 10% FBS and 100 U/ml penicillin and streptomycin (Cat# 15140–122; Gibco). The HEK293T cell line and its deviates were cultured in RMPI-1640 medium (Cat# 31800–022; Gibco) supplemented with 10% FBS and 100 µ/l penicillin and streptomycin (Cat# P4333; Sigma Chemical Co., Saint Louis, Missouri, USA). The normal human lung fibroblast WI-38 VA-13 subline 2RA (WI38–2RA) cells were purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (BCRC, Hsinchu, Taiwan). WI38–2RA cells were cultured in Minimum essential medium (MEM, Cat# 12000–022; Gibco) supplemented with 10% fetal bovine serum (FBS, Cat# 90708–085; VWR, Wayne County, PA, USA), 2 mM l-glutamine (Cat# A29168–01; Gibco), 1.5 g/l sodium bicarbonate (Cat# 144–55–8–131638; PanReac AppliChem ITW Reagents, Chicago, IL, USA), 0.1 mM non-essential amino acids (Cat# 11140–050; Gibco), and 1.0 mM sodium pyruvate (Cat# 11360–070; Gibco). All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C.
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Delivered dose cultivated at 37 °C under a mixture of 95% air and 5% CO₂.

Plasmids

pLAS2 is a lentiviral vector with the CMV promoter and was obtained from the RNAI core of Academia Sinica, Taiwan. We therefore inserted a GFP gene after the CMV promoter of the pLAS2 lentiviral plasmid (pLAS2-GFP). pcDNA3.1-SARS2-Spike is an expression plasmid carrying the wild-type (Wuhan strain) SARS-CoV-2 Spike protein with a C9 tag at its terminus and was a gift from Fang Li (Addgene plasmid #145032). We further modified this Spike-expressing plasmid with D614G plus N501Y (D614G/N501Y) mutations at Spike gene by PCR cloning and applied it to all this research for pseudovirus production and transfection experiments. Other Spike mutations were constructed based on pcDNA3.1-SARS2-Spike D614G/N501Y plasmid through restriction enzyme digestion or overlapping PCR, which included the deletion mutations with the indicated amino acids 586–978, 820–1273, and 14–685. The sequences of the plasmids constructed by PCR cloning were further validated through Sanger sequencing. The lenti-AE2 plasmid was a gift from Academia Sinica, Taipei, Taiwan.

Pseudovirus production

Pseudovirions were produced by the co-transfection of HEK293T cells with pCMV8.91 plasmid, a lentiviral plasmid encoding CMV-promoter-driven GFP gene (pLAS2-GFP), and a plasmid encoding SARS-CoV-2 Spike gene (pcDNA3.1-Spike D614G/N501Y) at the ratio 6.25 : 6.5 : 1.1 using PEI transfection (PEI MAX, MW 40,000, Polysciences, Warrington, PA, USA) following the manufacturer’s instructions. The supernatants were collected at 72 h post transfection and passed through a 0.45-μm filter. The SARS-CoV-2 S pseudovirions were further concentrated by sucrose centrifugation method (Jiang et al., 2015) and resuspended in RPMI medium supplemented with 10% FBS.

Cell viability

Cells (5 × 10³ cells/well) were seeded into 96-well culture plate dishes and incubated overnight. Cells were treated with GMI (0–1.2 μM) as indicated for 24-72 h. After incubation, cell viability was assessed by MTT assay (Cat # M5655; Sigma Chemical Co.) as described previously (Lin et al., 2021).

Immunoblotting analysis

Cells were treated with various concentration of GMI for indicated times. After that, cells were rinsed with PBS and harvested by scraping the cells into lysis buffer (Qiu et al., 2020) containing proteinase inhibitors (Cat# P8340; Sigma Chemical Co.). Whole cell lysates were harvested to determine the protein concentration by using a Bradford assay (Cat# 5000006; Bio-Rad, Hercules, California, USA). Cell lysate (20–30 μg) were then subjected to Western blot analysis which was conducted as previously described (Lin and Hsu, 2016). The antibodies against ACE2 (Cat# GTX101395), TMPRSS2 (Cat# GTX100743) and tubulin (Cat# GTX112141) were purchased from GeneTex (Hsinchu, Taiwan). The expression of tubulin was used as an internal control.

Delivered dose (μg / kg) = Concentration of GMI in air (μg/l) × Respiratory minute volume (l/min) × Duration of exposure (min) / Bodyweight (kg)

Pseudovirus infection

To evaluate the transduction efficiency of Spike pseudovirus, 2500 HEK293T/ACE2 cells were seeded in 96-well plates per well and treated with various concentrations of GMI at different time periods according to the experiment. After pre-treatment, the culture medium was removed and replenished with fresh medium containing GFP-encoding Spike pseudovirus with or without GMI as indicating in each experiment. The transduction efficiency of each sample was visualized 48 h post transduction by fluorescence microscopy (Zeiss, Jena, Germany) and the percentage of GFP-positive cells was quantified by flow cytometry (FACScalibur, BD, Hampton, NH, USA).

Pull down assay

HEK293T cells were at first transfected with the indicated SARS-CoV-2 Spike-encoding plasmids for 72 h and then exposed to GMI (0.3 μM) at 37 °C for 1 h. Subsequently, the cells were washed with PBS three times and lysed in lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40, and 2 mM EDTA) at 4 °C under rotation at 100 rpm for 1 h by using a rolling device (Model: RM-2 l, ELMI Ltd., Riga, Latvia). The lysate was centrifuged at 21,000g for 10 min, and the clarified lysates were collected and subjected to immunoprecipitation using a Dynabeads Protein G Immunoprecipitation kit (Cat# 10007D; Invitrogen, Villeurs, Lithuania) following the manufacturer’s instructions. We applied a rabbit polyclonal antibody against the SARS-CoV-2 Spike protein (obtained from Academia Sinica) and a mouse monoclonal antibody against the His-tagged-GMI (Cat# MA1–21315, Clone HIS.H8, Thermo Scientific, Rockford, IL, USA) for immunoprecipitation. After the incubation of lysates with antibody-loaded Protein G Dynabeads at room temperature for 1 h, the beads together with the pulled-down proteins were collected using a magnet and washed four times with washing buffer. The immunoprecipitates together with Dynabeads Protein G were then eluted in Laemmli buffer for 10 min at 95 °C and separated on SDS-polyacrylamide gels for Western blotting.

Nebulized liquid GMI aerosols on mice model

The 6-8 weeks male C57BL/6 mice were purchased from National Laboratory Animal Center (NLAC, Taipei, Taiwan). The experiment was carried out in a controlled atmosphere with a 12 h light and 12 h dark cycle, which followed the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) of NYCU (IACUC Approval NO: 1101212). GMI (100 μg) was dissolved in physiologic saline (1 ml). The GMI was provided to mice by using the nebulizer (Aerogen AG-AP1000, Aerogen Ltd. Galway, Ireland) with a flow rate 0.25 ml/min. The mice were divided into three groups, including control group (CTL), one time exposure of GMI (Exp. 1) and two times exposure of GMI (Exp. 2). The mice were put into the 23 × 11 × 11 cm³ cages (about 2.8 l) and inhaled the aerosol of GMI in the cage for 30 min. After 6 h, the mice were sacrificed and lung tissues were harvested and stored at −80 °C for future investigation. To calculate the delivered dose of GMI, the equation of below was used (Alexander et al., 2008). The concentration of GMI in air (μg/l) was 100 μg/2.8 l cage volume. The respiratory minute volume was calculated according to the formula and was calculated as 0.021 l/min in mice. The average body weight of the mice was 0.02 kg. The delivered doses for the 30 min exposure groups were 1125 μg/kg.

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Respiratory minute volume (l/min) = 0.608 × bodyweight (Kg)^0.832

Statistical analysis

GraphPad Prism8 was used for statistical analysis. The experiments were conducted three times or as indicated, and all data are expressed as mean ± SD. Statistical differences between the control and experimental groups were examined by t-test and one-way ANOVA. p-values < 0.05 were considered statistically significant.

Results and discussion

GMI does not exhibit the cytotoxic effect on HEK293T/ACE2 cells but abolish SARS-CoV-2 pseudovirus infection

ACE2 is the pivotal receptor for SARS-CoV-2 infection (Jia et al., 2021). To examine the efficacy and potential mechanisms of GMI in the prevention of SARS-CoV-2 infection, we established an ACE2 over-expressing HEK293T cells, noted HEK293T/ACE2 (Supplementary Fig. 1A). Initially, we examined the cytotoxic effects of GMI on HEK293T/ACE2 cells. Using the MTT assay, we found that GMI did not significantly inhibit cell viability of HEK293T/ACE2 in 24 to 72 h of treatment (Fig. 2A). The cytotoxic concentration 50 (CC_{50}) of GMI in the 24, 48 and 72 h treatment was more than 1.2 μM. These results indicated that GMI did not affect cell viability of HEK293T/ACE2 cells.

Next, we examined whether GMI could affect SARS-CoV-2 infection. The SARS-CoV-2 Spike pseudovirus expressing GFP protein was applied (Condor Capcha et al., 2020) for evaluating the effects of GMI on SARS-CoV-2 infection. The infectivity of Spike pseudovirus can be analyzed by fluorescence microscopy and flow cytometry. Initially, we demonstrated that SARS-CoV-2 Spike pseudovirus expressing GFP protein effectively infected HEK293T/ACE2 cells but not HEK293T cells (Supplementary Fig. 1B). Next, HEK293T/ACE2 cells were treated with GMI and concomitantly infected with SARS-CoV-2 Spike pseudovirus for 48 h (Fig. 2B). As expected, GMI effectively reduced SARS-CoV-2 pseudovirus infection of HEK293T/ACE2 cells by showing far less GFP-positive cells (Fig. 2C). Simultaneously, we used flow cytometry to quantify the percentage of GFP-positive cells in each sample and found that GMI significantly reduced the transduction rate of Spike pseudovirus to HEK293T/ACE2 cells by up to 80% while using GMI at the concentration of 0.6 μM (Fig. 2D). In addition, we found that GMI reduced SARS-CoV-2 pseudovirus infection of lung epithelial carcinoma Calu-3 cells (Supplementary Fig. 2). Together, these findings suggest that GMI may act as a potential agent to inhibit SARS-CoV-2 infection.

GMI binds to S2 domain of Spike protein and slightly reduces interaction between ACE2 and Spike

Currently, blocking the interaction between SARS-CoV-2-Spike (S) and ACE2 is a potential therapeutic direction in treating SARS-CoV-2 infection (Zamorano Cuervo and Grandvaux, 2020). To validate the role of GMI on virus infection, we initially hypothesized that GMI may block SARS-CoV-2-S/ACE2 interactions. We conducted the ACE2/Spike binding assay and found that SARS-CoV-2-S/ACE2 interactions were slightly decreased by 20% after the incubation of GMI at 1.2 μM with either SARS-CoV-2-S or ACE2 (Figs. 3A-B). In addition, to examine whether GMI could attract the virus and reduce its infection rate, virus was pretreated with GMI for 10 min and then harvested by centrifugation (Fig. 3C). Similar to the results of the in vitro binding assay, we found that the pretreatment of GMI on virus reduced the infection rate by 20% (Figs. 3D-E). These findings led us to investigate whether GMI might interact with SARS-CoV-2-S. Therefore, the purified Spike protein was co-incubated with GMI in vitro to examine the interaction between GMI and Spike protein by the immunoprecipitation of Spike. As expected, we found that GMI could interact with Spike protein (Supplementary Fig. 3). We next constructed the expression plasmids encoding...
Fig. 3. GMI interacted with S2 domain of SARS-CoV-2-S to interfere the interaction between ACE2 and SARS-CoV-2-S. (A) ACE2 proteins were pre-incubated with GMI for 1 h. After the incubation, the solution was added into the Spike-coating well for 2.5 h to detect the interaction between ACE2 and Spike. (B) GMI was added to the Spike-coating well for 1 h. After the incubation, ACE2 protein was added into the well for 2.5 h to detect the interaction between ACE2 and Spike. (C) Schematic design of the in vitro GMI (0.3 μM)-treated SARS-CoV-2 pseudovirus infection experiment. (D-E) The infected cells were GFP positive observed by fluorescence microscopy (D) and the percentage of GFP-positive cells in each sample was quantified by flow cytometry (E). The data were representative of three separated experiments and were presented as mean ± SDs; the error bars indicated SD. Significant differences were noted. (F) Up-left panel: different constructs of SARS-CoV-2-S. Up-right and bottom panels: representative immunoblots of HEK293T/ACE2 cells transformed with different mutant constructs of SARS-CoV-2-S and incubated with GMI (0.3 μM) for 1 h. The pull-down assay was shown in Materials and methods section. (* p < 0.05 compared to the control group).
different SARS-CoV-2-S mutants and overexpressed on the HEK293T cells. By using co-immunoprecipitation assay, we found that GMI could physically interact with the full-length Spike protein whereas this interaction was compromised by using the Spike protein with deletion at S2 domain (Fig. 3F). It is well-known that receptor-binding domain (RBD) in S1 subunit of SARS-CoV-2 is a key for binding to ACE2 (Lan et al., 2020). However, we found that GMI interacted with S2 subunit of SARS-CoV-2, suggesting that GMI could not directly affect the interaction between Spike and ACE2. The functions of GMI binding to S2 subunit of SARS-CoV-2 need to be examined in the future. Taken together, GMI-inhibited SARS-CoV-2 pseudovirus infection did not fully depend on attacking the virus but might due to the slight interference in SARS-CoV-2/S/ACE2 interactions via binding to S2 domain of SARS-CoV-2-S or other mechanisms.

GMI reduces ACE2 levels via the induction of protein degradation in HEK293T/ACE2 cells

To examine the potential mechanism of GMI prevented SARS-CoV2 pseudovirus infection, we investigated whether GMI could directly affect the protein levels of ACE2 and TMPRSS2 on HEK293T/ACE2 cells. As shown in Fig. 4A, we found that GMI reduced expressions of ACE2 in short and long-term treatment; however, GMI did not affect the TMPRSS2 levels. These results suggested that GMI may specifically target on ACE2 in HEK293T/ACE2 cells. To further inspect the mechanism of GMI on the regulation of ACE2 expression, we initially examined whether GMI induced the activation of Adam17, which could cleave the ACE2 (Heurich et al., 2014). However, GMI did not enhance the activity of Adam17 in lung cancer cells (data not shown). In addition, we found that GMI did not affect the mRNA levels (data not shown). We thus hypothesized that GMI may interfere the stability of ACE2. We used the cycloheximide (CHX), a ribosome inhibitor, to block the protein synthesis, and found that the co-treatment of GMI and CHX effectively reduced ACE2 levels compared to CHX individual treatment (Fig. 4B). The findings suggested that GMI may induce ACE2 degradation.

Next, we examined the protein degradation pathways in GMI-treated HEK293T/ACE2 cells. Previous studies showed that proteasome contributes to ACE2 degradation (Liu et al., 2014; Shen et al., 2020; Wang et al., 2021). We therefore investigated whether GMI-induced ACE2 degradation was related to proteasome pathway. Using the proteasome inhibitor, MG132, we found that MG132 could decrease GMI-reduced ACE2 levels in the short and long-term treatment (Fig. 4C). In addition, evidence shows that angiotensin-II reduces ACE2 by stimulation of lysosomal degradation pathway (Deshorts et al., 2014). Using the lysosome inhibitors, Bafl, to block the lysosome activity, we also found that ACE2 levels were maintained in HEK293T/ACE2 cells which were treated with GMI (Fig. 4D). These results indicated that GMI may disturb the stability of ACE2, which could lead to ACE2 degradation. Herein, we found that GMI could induce ACE2 degradation via proteasome and lysosome pathway. Wang et al. also demonstrated that benzo(a)pyrene (BaP, a carcinogen) could trigger ACE2 degradation via proteasomal and lysosomal pathways (Wang et al., 2021). Evidence shows that membrane proteins are usually degraded due to ubiquitination-dependent endocytosis (Marmor and Yarden, 2004; Weinberg and Drubin, 2014). This finding indicate that GMI may activate two protein degradation systems, causing endocytosis and degradation of ACE2.

Increasing evidence shows that coronaviruses enter host cells via binding to ACE2 and triggering clathrin or lipid raft-dependent endocytosis (Bian and Li, 2021). We thereby examined the endocytosis pathways in GMI-induced ACE2 degradation. Initially, dynasore, a dynamin inhibitor, was chosen to inhibit clathrin-dependent endocytosis (Kirchhausen et al., 2008). As shown in Fig. 4E, we found that...
dynasore increased ACE2 levels and rescued GMI-reduced ACE2 expression. Next, we used the methyl-β-cyclodextrin (MβCD) to disrupt lipid raft. We found that MβCD increased ACE2 levels for the long-term treatment (Fig. 4F). Specifically, MβCD rescued GMI-reduced ACE2 levels (Fig. 4F). We also examined the interaction between GMI and ACE2. However, we did not identify the complex of ACE2/GMI by using the immunoprecipitation assay (data not shown). Together, these results suggest that GMI downregulated expression of ACE2 via two steps: 1. GMI induced clathrin and lipid raft-dependent endocytosis of ACE2; 2. ACE2 was then degraded via lysosomal and proteasomal pathways.

**Discontinuous GMI exposure maintains the inhibition rate of SARS-CoV-2 pseudovirus infection**

To analyze the efficacy of GMI on regulating ACE2 levels and inhibition rate of SARS-CoV-2 pseudovirus infection, we conducted the discontinuous GMI exposure experiments and examined the cell viability of HEK293T/ACE2 cells. HEK293T/ACE2 cells were exposed to GMI for 48 h and examined the cell viability after removing GMI. As shown in Fig. 5A, we found that when GMI was given for 48 h, removing GMI (1.2 μM) from the HEK293T/ACE2 cells slightly inhibited the cell viability approximately 10%. Therefore, we further examined the

![Fig. 5. Discontinuous GMI exposure preserved inhibition of the SARS-CoV-2 pseudovirus infection. (A) The cell viability HEK293T/ACE2 cells were treated with GMI at different time points and the cell viability was measured by MTT assay. (B) The expressions of ACE2 protein were determined by Western blot. Tubulin was used as internal control. The intensities of the bands of ACE2 were quantified by ImageJ. Data were presented as the mean ± SD; error bars indicated SDs. Significant differences were shown (**p < 0.001 compared to the control (CTL) group; #p < 0.05 compared to GMI treatment for 48 h). (C) The schematic design for the continuous and discontinuous GMI treatment. (D-E) The infected cells were GFP positive and were observed by fluorescence microscopy (E) and quantified by flow cytometry (D). Data were presented as the mean ± SD; error bars indicated SDs. Significant differences were shown (**p < 0.001 compared to the control (CTL) group; #p < 0.05 compared to each discontinuous GMI treatment group).](image-url)
treatment condition on ACE2 levels. As expected, GMI (0.6 μM) dramatically reduced ACE2 level (Fig. 5B). Specifically, GMI substantially downregulated ACE2 levels by 80% after 48 h treatment. Interestingly, if GMI was removed after 48 h treatment, ACE2 would slightly rise after another 24 and 48 h (Fig. 5B). However, the ACE2 levels still cannot return to 100% after GMI removal. These results indicate that after GMI was removed from the host cells, ACE2 levels was not quickly recovered to normal levels, and suggest GMI treatment can prolong the efficacy of protection against SARS-CoV-2 infection. Therefore, we conducted the experiments to examine the efficacy of GMI on infection after removing GMI (Fig. 5C). After 48 h of GMI treatment, GMI was removed and at the same time the SARS-CoV-2 pseudovirus was given on the HEK293T/ACE2 cells for 48 h. We found that GMI effectively inhibited the virus infection in a concentration-dependent manner (Exp.1 vs CTL; Figs. 5D-E). Moreover, with the continuous treatment of GMI SARS-CoV-2 pseudovirus infection, the inhibition rate of infection increased to more than 80%. Specifically, the continuous treatment of GMI significantly reduced SARS-CoV-2 pseudovirus infection compared to discontinuous administration (Exp. 2 vs Exp. 1; Figs. 5D-E). Taken together, GMI exhibits the great efficacy of the inhibition of SARS-CoV-2 pseudovirus infection.

GMI reduces ACE2 expression on lung WI38–2RA cells in vitro and lung tissue in vivo

As shown in the above findings, we found that GMI effectively downregulated ACE2 expression via induction of protein degradation system. Therefore, we examined the effects of GMI on normal human lung cells by inspecting the cytotoxic effects of GMI on lung WI38–2RA cells. Using the MTT assay, we found that GMI did not significantly inhibit cell viability of WI38–2RA after 48 and 72 h treatment (Fig. 6A). The CC50 of GMI in the 48 and 72 h treatment was 2.4 and 1.9 μM, respectively. Moreover, we found that GMI did not affect the cell viability of lung fibroblast MRC-5 and alveolar macrophage MH-S (data not shown). Together, GMI did not affect cell viability of normal lung cells.

Next, we investigated whether GMI could affect the protein levels of ACE2 in WI38–2RA cells. As shown in Fig. 6B, the data showed that GMI reduced expressions of ACE2 in the short and long-time treatment; specifically, GMI dramatically downregulated ACE2 after 24 h.

Fig. 6. GMI downregulated expression of ACE2 in WI38–2RA cells in vitro and lung tissue of mice in vivo. (A) The viability of WI38–2RA cells treated with GMI was evaluated. Each GMI-treated group was normalized by the control group. (B) The ACE2 levels of WI38–2RA cells treated with GMI were evaluated by Western blot. (C-F) WI38–2RA cells were pretreated with DMSO/ddH2O (vehicle control) or MG132 (10 μM; C)/Bafilomycin A1 (BafA1; 20 nM; D)/dynasore (200 μM; E)/MβCD (20 mM; F) for 30 min, followed by incubation with GMI (0.6 μM) for 24 h and their ACE2 expression levels were evaluated. (G) Scheme for mouse receiving GMI via inhalation method. (H) The expressions of ACE2 protein in lung tissue were determined by Western blot. Tubulin was used as internal control. Each number (#1, #2, #3) represents the data collected from one single mouse. The data were representatives of more than three separated experiments and were presented as mean ± SDs; the error bars indicated SD. Significant differences were noted (*** p < 0.001).
treatment. These results suggested that GMI may specifically target on ACE2 in WI38–2RA cells. Similarly, to dissect the mechanism of GMI on regulation of ACE2 expression, we examined whether GMI induced ACE2 degradation via degradation system. As shown in Figs. 6C–D, either MG132 or BafA1 effectively rescued GMI-reduced ACE2 levels. Moreover, dynasore and MJiCD rescued GMI-induced ACE2 levels (Figs. 6E–F). These results are consistent to the above findings that GMI may be a potential agent for induction of ACE2 degradation.

To examine the effects of GMI on ACE2 expression of lung tissue in mice, mice were exposed to GMI by using spray method (inhalation, Fig. 6G). Two types of GMI exposure were performed. In the type one (Exp. 1), mouse was exposed to GMI for 30 min and sacrificed after 6 h. In the type two (Exp. 2), mouse was exposed to GMI for 30 min. After 24 h, the mouse was exposed to GMI for 30 min again and then sacrificed after 6 h. As expected, we found that ACE2 levels were dramatically downregulated by more than 60% in the lung tissue after the mice inhaling GMI (Fig. 6H). These findings indicated that GMI may be used as a nasal spray agent to reduce the expression of ACE2 in lung tissues in vivo.

Currently, vaccines have been developed for preventing COVID-19. However, the coverage and efficacy of the vaccine remain to be further improved. Development of additional strategies to temporarily reduce SARS-CoV-2 infection may be an important issue. In this study, we attempted to dissect the effects of GMI on preventing SARS-CoV-2 infection. We established an ACE2 overexpressing HEK293T cells and the SARS-CoV-2 Spike pseudovirus to evaluate the effects of GMI on SARS-CoV-2 infection. Alternatively, we demonstrated that GMI did not inhibit VSV-G pseudotyped virus infection (Supplementary Fig. 4), suggesting that GMI may specifically target on SARS-CoV-2 infection. Currently, multiple potential anti-SARS-CoV-2 strategies such as receptor binding, entry/fusion, and replication have been dissected (Hu et al., 2021). Unlike the small compound to target replication-related enzymes, GMI is a fungal protein which may interfere with the interaction between SARS-CoV-2 and host cells. We therefore examined whether GMI blocked the binding of ACE2 to SARS-CoV-2-S. However, GMI slightly abolished the SARS-CoV-2-S/ACE2 interactions by 20%, suggesting that GMI may interact with SARS-CoV-2-S or ACE2. We found that GMI did not bind to ACE2 (data not shown). In contrast, GMI could interact with S2 subunit of SARS-CoV-2-S. Previous study showed that a carbohydrate-binding protein (noted FRIL) from Lablab purpureus effectively blocks the infections of SARS-CoV-2 via binding to N-glycan of glycoprotein (Liu et al., 2020). Specifically, FRIL interacts with virus to form aggregates may prevent virus entry or trap the virus in the late endosome. Whether GMI exhibits the similar functions of FRIL needs to be explored in the future. In parallel, targeting on the receptors of SARS-CoV-2-S on host cells may be an intervention strategy (Shang et al., 2020). ACE2 is a well-known membrane protein controls SARS-CoV-2 infection (Shang et al., 2020). We found that GMI effectively reduced ACE2 levels on the host cells. Specifically, GMI triggered the endocytosis and degradation of ACE2 on host cells, resulting in reducing the SARS-CoV-2 pseudovirus infection. Until now, less study focused on targeting ACE2 degradation as a novel strategy for preventing the SARS-CoV-2 infection (Su et al., 2021). Herein, we provide a novel fungal protein, GMI, which reduced SARS-CoV-2 pseudovirus infection in vivo. Moreover, we found that GMI could reduce ACE2 levels in lung tissue of mice. These findings indicate that GMI reduced expression of ACE2 in vitro and in vivo, suggesting that GMI may be used as a potential agent for reducing SARS-CoV-2 infection. Whether GMI could be a drug for the treatment of COVID-19 in clinics needs to further be dissected.

Conclusion

This study identified the efficacy of a fungal protein, GMI, for preventing SARS-CoV-2 infection and further dissect the potential mechanisms. To the best of our knowledge, this is the first evidence indicating that GMI effectively inhibited SARS-CoV-2-S pseudovirus infection. Moreover, we found that GMI could bind to S2 domain of SARS-CoV-2-S and slightly interfere the interaction between Spike and ACE2. Importantly, we demonstrated that GMI downregulated ACE2 levels on the host cells. Specifically, GMI promoted clathrin and lipid raft-dependent ACE2 endocytosis, resulting in induction of ACE2 degradation. These significant results suggest that GMI may be a promising prevention agent to alleviate SARS-CoV-2 infection.

CRediT authorship contribution statement

H. Yeh, D.-N K. Vo, Z.-H. Lin, H.-P T. Ho, and W.-I. Qiu performed experiments; W.-J. Hua edited manuscript. M.-H. Tsai designed pseudovirus study and wrote the manuscript. T.-Y. Lin provided conception, designed research, analyzed data, wrote and revised the manuscript; all of authors approved final version of manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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References

Abbasi, J., 2021. COVID-19 mRNA vaccines blunt breakthrough infection severity. JAMA 326, 473.
Alexander, D.J., Collins, C.J., Coombs, D.W., Gilkinson, I.S., Hardy, C.J., Healey, G., Karantabias, G., Johnson, N., Karlsson, A., Kilgour, J.D., McDonald, P., 2008. Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. Inhal. Toxicol. 20, 1179–1189.
Bian, J., Li, Z., 2021. Angiotensin-converting enzyme 2 (ACE2): SARS-CoV-2 receptor and RAS modulator. Acta Pharm. Sin. B 11, 1–12.
Condor Capcha, J.M., Lambert, G., Dykxhoorn, D.M., Salerno, A.G., Hare, J.M., Whitt, M.A., Palvera, S., Jayaweera, D.T., Sheradeh, L.A., 2020. Generation of SARS-CoV-2 Spike pseudotyped virus for viral entry and neutralization assays: a 1-week protocol. Front. Cardiovasc. Med. 7, 618651.
Deshotels, M.R., Xia, H., Siriramala, S., Lazarriques, E., Filippeau, C.M., 2014. Angiotensin II mediates angiotensin converting enzyme type 2 internalization and degradation through an angiotensin II type 1 receptor-dependent mechanism. Hypertension 64, 1368–1375.
