Original article

GB-2 inhibits ACE2 and TMPRSS2 expression: In vivo and in vitro studies

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ARTICLE INFO

ABSTRACT

After the first case of Coronavirus disease 2019 (COVID-19) was reported in Wuhan, COVID-19 has rapidly spread to almost all parts of world. Angiotensin converting enzyme 2 (ACE2) receptor can bind to spike protein of SARS-CoV-2. Then, the spike protein of SARS-CoV-2 can be cleaved and activated by transmembrane protease, serine 2 (TMPRSS2) of the host cells for SARS-CoV-2 infection. Therefore, ACE2 and TMPRSS2 are potential antiviral targets for treatment of prevention of SARS-CoV-2 infection. In this study, we discovered that 10−250 µg/mL of GB-2, from Tian Shang Sheng Mu of Chiayi Puzi Peitian Temple, can inhibit *ACE2* mRNA expression and ACE2 and TMPRSS2 protein expression in HepG2 and 293 T cells without cytotoxicity. GB-2 treatment could decrease ACE2 and TMPRSS2 expression level of lung tissue and kidney tissue without adverse effects, including nephrotoxicity and hepatotoxicity, in animal model. In the compositions of GB-2, we discovered that 50 µg/mL of theaflavin could inhibit protein expression of ACE2 and TMPRSS2. Theaflavin could inhibit the mRNA expression of *ACE2*. In conclusion, our results suggest that GB-2 and theaflavin could act as potential compounds for ACE2 and TMPRSS2 inhibitors in the further clinical study.

1. Introduction

Since the outbreak of coronavirus disease 2019 (COVID-19) in Wuhan, China, more than 10 million people have been infected and 50,000 patients have died worldwide. This global infectious disease was induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a coronavirus with a spike protein. The receptor-binding domain of the spike protein on the viral envelope of SARS-CoV-2 can directly bind to angiotensin-converting enzyme 2 (ACE2), a membrane-associated enzyme [1]. The SARS-CoV-2 spike protein can be cleaved and activated by transmembrane proteases, including serine 2 (TMPRSS2) and furin in host cells, thus facilitating the entry of SARS-CoV-2 into host cells [2–5]. Moreover, the spike protein of SARS-CoV-2 is highly similar to SARS-CoV but has a higher binding affinity to the ACE2 receptor when compared with SARS-CoV [2–4]. The high affinity of SARS-CoV-2 to ACE2 may promote human-to-human transmission. Therefore, diminishing the protein expression of ACE2 and TMPRSS2 in host cells could be an effective strategy for the prophylaxis and treatment of SARS-CoV-2 infection.

Studies have identified high ACE2 expression in the lungs, kidney, esophagus, colon, heart, brain, and other organs [6–9]. TMPRSS2 is an androgen-responsive serine protease and is expressed in several tissues and organs, including the lungs, skin, and urogenital tract [10,11]. Organs with a relatively high number of ACE2 and TMPRSS2-expressing cells should be considered as potential SARS-CoV-2 infection sites. Studies have demonstrated the presence of SARS-CoV-2 viral particles in kidney and lung endothelial cells [12,13]. The entry of SARS-CoV-2 into endothelial cells can induce damage to these cells, with subsequent inflammation and prothrombin generation. Then, the COVID-19 patients suffered from severe syndromes, including acute respiratory distress syndrome and acute kidney injury [14]. Therefore, targeting both ACE2 expression and TMPRSS2 expression in lung and kidney cells could be a potential antiviral strategy for the prevention or treatment of SARS-CoV-2 infection [4,15–17].

Several natural compounds have been screened as therapeutics for COVID-19 treatment. However, most studies have used only in silico

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molecular docking for screening [18]. The in vivo effects and the mechanism underlying the activities of these natural compounds in blocking SARS-CoV-2 entry into host cells remain unclear. GB-2, the formula from Tian Shang Sheng Mu of Chiayi Puzi Peitian Temple, was used for the prophylaxis of SARS-CoV-2 infection in Taiwan. In this study, we investigated the in vivo and in vitro effects of GB-2 and theaflavin, the index compound of *camellia sinensis* var. assamica extract, on the protein and mRNA expression of ACE2 and TMPRSS2.

2. Methods and materials

2.1. Cell culture and treatment

HepG2 cells (human hepatocellular carcinoma cell line) and 293 T cells (human embryonic kidney cell line) were obtained from the Bioresource Collection and Research Center, Taiwan. Both HepG2 cells and 293 T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen Corp., Carlsbad, CA), supplemented with 10 % FBS at 37℃ and 5% CO2. The GB-2 formula was designed from Tian Shang Sheng Mu of Chiayi Puzi Peitian Temple. The compositions of GB-2 included dry root of *glycyrrhiza uralensis* Fisch. ex DC. (10 g, brought form Chang Gung Memorial Hospital, Taiwan) and *camellia sinensis* var. assamica (black tea, 25 g, brought form Chang Gung Memorial Hospital, Taiwan). The formula was soaked in 2000 mL water and boiling hot water for 25 min in thermal flasks, respectively. The sample was filtered with filter paper to remove particulate matter. The water extracts were evaporated through reducing pressure to obtain viscous masses (GB-2: 6 g). These samples were stored at −80 °C. For all experiments, final concentrations of the tested compound were prepared by diluting the stock with water. Before treatment, HepG2 cells and 293 T cells were cultured to 60–70 % confluence. Then, cultured medium was replaced with fresh medium containing indicated compounds in water at the indicated concentrations. HepG2 cells and 293 T cells treated with water were used as controls. HepG2 cells and 293 T cells without treatment were used as blank control.

2.2. XTT assay

HepG2 cell or 293 T cell lines were cultured at a density of 1×10^3 per well of 96-well plates, in DMEM with 10 % FBS. Once attached, the cultured medium was replaced with fresh medium with 10 % FBS. HepG2 cell or 293 T cell were then treated with indicated drugs for indicated hours; and absorbance were measured using the XTT assay kit (Roche, catalog number: 11,465,015,001) depending on the manufacturer's instructions. The XTT formazan complex was quantitatively measured at 492 nm using an ELISA reader (Bio-Rad Laboratories, Inc.).

2.3. Western blot analysis

HepG2 or 293 T cellular extracts after indicated treatments were collected and prepared according to previously studies for Western blot analysis [19,20]. Equal amounts of protein in each groups were fractionated on a 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then, the membranes were blocked by 5% nonfat dried milk for 30 min. The membranes were incubated in primary indicated antibody for 12 h at room temperature. These primary antibodies included: anti-ACE2 antibody (Cell Signaling, ratio: 1:1000), anti-TMPRSS2 antibody (Abcam, ratio: 1:1000) and anti-GAPDH antibody (Santa Cruz, IB: 1:10000). Both the primary antibodies and the secondary antibodies were diluted with 1% nonfat dried milk in 0.1 % TBST (Tris-Buffered Saline Tween-20). Next, the membranes were washed by 0.1 % TBST and incubated in horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (Santa Cruz, ratio: 1:5000) which were diluted with 1% nonfat dried milk in 0.1 % TBST for one hour at room temperature. The protein signals were detected by chemiluminescence through the Super Signal

substrate (Pierce, catalog number: 34,087).

2.4. Quantification of protein level

The images of each bands from Western's blotting were analyzed by AlphaEase®FC software depending on the manufacturer's instructions for quantification of protein level. The densities of bands in each group were automatically calibrated by subtracting the background. The density of the group without treatment was used as the standard to calculate the ratio value of the other treated groups.

2.5. Quantitative real time PCR

Quantitative Real time PCR were performed as described previous studies [19,20]. Total RNA of treated HepG2 cells or 293 T cells were collected and extracted through the illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, catalog number: 25−0500). Reverse transcription was performed by the Superscript first strand synthesis kit (Invitrogen, catalog number: 11,904,018). Quantitative real-time PCR analyses using the comparative CT method were performed on an ABI PRISM 7700 Sequence Detector System using the SYBR Green PCR Master Mix kit (Perkin Elmer, Applied Biosystems, Wellesley, MA, USA). Following initial incubation at 50 °C for 2 min and 10 min at 95 °C, amplification was performed for 40 cycles at 95 °C for 20 s, 65 °C for 20 s and 72 °C for 30 s. Primers used were: *ACE2* forward, 5′- TCC ATT GGT CTT CTG TCA CCCG-3′ and *ACE2* reverse, 5′- AGA CCA TCC ACC TCC ACT TCTC-3′. *GAPDH* forward, 5′-TGC ACC ACC AAC TGC TTAGC-3′ and *GAPDH* reverse, 5′-GGC ATG GAC TGT GGT CATGA-3′. *GAPDH* was used as the housekeeping gene for data normalization.

2.6. Mouse model

All procedures involving mouse model were approved by Animal Care and Use Committee (Approval number 2,017,081,601) of Chang Gung Memory Hospital. 10 male C57BL/6 mice (18–20 g) aged 5–7 weeks were obtained from BioLASCO Taiwan Co., Ltd. for the animal model. Mice were randomized into 2 groups and 5 mice per each group. One group was treated with vehicle (water) and the other group was treated with 200 mg/kg/day GB-2 everyday by oral administration. Mouse weight were measured every 1–2 days for 1 week. Following 1 week, the mice were sacrificed for autopsy under carbon dioxide gas method. The blood specimens were submitted for serum creatinine, GOT (aspartate aminotransferase) and GPT (alanine aminotransferase) level after sacrificed.

2.7. Immunohistochemistry (IHC) assessment

IHC assessment was performed as described previous study [21]. Lung and kidney tissue specimens from mice underwent fixation with 4% formalin, paraffin embedding, sectioning and staining with ACE2 primary antibody (Bioss Antibodies, ratio: 1:100) or TMPRSS2 primary antibody (Abcam, ratio: 1:1000). Peroxidase-linked goat anti-rabbit secondary antibodies and the rabbit probe HRP labeling kits were used for IHC assessment (BIOTNA, catalog number: TAHC03D). Photomicrographs were observed using Nikon TE3000 microscope. ImageJ (1.50d, USA) system was used to quantify integrated optical density per stained area (IOD/area) for ACE2 and TMPRSS2 staining.

2.8. Statistical analyses

All values were the means ± standard error of mean (SEM) of the replicate samples (n = 3–6, depending on the experiment). These experiments were repeated by a minimum of three times. Differences between two groups were assessed using the unpaired two-tailed Student's *t*-test or by ANOVA if more than two groups were analyzed. For testing the significance of pairwise group comparisons, the Tukey test was used

as a post-hoc test in ANOVA. For all comparisons, *P* values of < 0.05 were considered statistically significant. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for all calculations.

3. Results

3.1. Effect of GB-2 on growth on different cell lines

A previous study used 293 T cells as a model of SARS-CoV-2 entry [22]. Moreover, another study applied HepG2 cells exhibiting high ACE2 protein expression as a model of SARS-CoV or SARS-CoV-2 entry [23]. To investigate the effect of GB-2 on ACE2 expression, we used both HepG2 cells and 293 T cells as models in our study. First, we conducted XTT assay to investigate the cytotoxicity of GB-2 against HepG2 cells and 293 T cells. Our results indicated that GB-2 administered over a concentration range of 10–250 μg/mL after the indicated hours could not inhibit the proliferation of the HepG2 cells (Fig. 1A) or 293 T cells (Fig. 1B). These data suggest that 10–250 μg/mL of GB-2 did not have significant cytotoxicity against the growth of these cell lines.

3.2. Effect of GB-2 on ACE2 and TMPRSS2 expression in different cell lines

Second, we investigated the effect of GB-2 on *ACE2* mRNA expression in host cells. The results demonstrated that GB-2 significantly diminished *ACE2* mRNA expression in the HepG2 cells (Fig. 2A) and 293 T cells (Fig. 2B) after 24 h of treatment. We subsequently explored the effect of GB-2 on the protein expression of ACE2 and TMPRSS2. The results indicated that GB-2 inhibited the protein expression of ACE2 and TMPRSS2 in HepG2 cells (Fig. 2C) and 293 T cells (Fig. 2D) in a dose-dependent manner after 24 h. These results suggest that 10–250 μg/mL of GB-2 can inhibit the protein expression of ACE2 and TMPRSS2 and

the mRNA expression of *ACE2* in these cell lines.

3.3. In vivo effect of GB-2 in a mouse model

To investigate the in vivo effect of GB-2, we used mice as our animal model. The mice were orally administered 200 mg/kg of GB-2 daily. After 1 week of GB-2 treatment, we observed no mouse death and no significant alteration in mouse activity or body weight (Fig. 3A). Furthermore, serum creatinine, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase levels were not significantly increased in the GB-2 group compared with the control group (Fig. 3B-D), suggesting GB-2 have no significant nephrotoxicity or hepatotoxicity.

As mentioned, the lung and kidney are critical organs with relatively high ACE2 expression levels and potential targets of SARS-CoV-2 [6,7, 24,25] ; accordingly, we investigated the effect of GB-2 on the protein expression of ACE2 and TMPRSS2 in the tissues of both organs. After 1 week of GB-2 treatment (200 mg/kg, oral administration), immunohistochemistry (IHC) data obtained for lung (Fig. 4A, B) and kidney (Fig. 4C, D) tissues revealed that the expression levels of both ACE2 and TMPRSS2 were markedly diminished in the GB-2 group compared with the control group. These results indicate that GB-2 may block ACE2 and TMPRSS2 expression in lung and kidney tissues without adverse effects, including nephrotoxicity and hepatotoxicity.

3.4. Effect of theaflavin on ACE2 and TMPRSS2 expression

The *camellia sinensis* var. assamica extract is the major component of GB-2. We investigated the effect of theaflavin (Fig. 5A), the index compound of *camellia sinensis* var. assamica extract, on 293 T cell lines. We tested the potential cytotoxicity of theaflavin administered over a concentration range of 10–50 μg/mL against 293 T cells. At 50 μg/mL, theaflavin exhibited mild cytotoxicity against the growth of 293 T cells in 24 h (Fig. 5B). However, only the theaflavin concentration of 50 μg/mL inhibited the protein expression of ACE2 and TMPRSS2 in 293 T cell lines in 24 h (Fig. 5C). These results suggest that higher concentrations ($\geq$50 μg/mL) of theaflavin can inhibit the protein expression of ACE2 and TMPRSS2 in 293 T cell lines.

4. Discussion

In 2003, ACE2 was identified as a receptor for SARS-CoV infection and transmission [26]. Some studies reported the distribution of high ACE2 expression in some organs, including the intestines, kidneys, heart, and lungs [6–8]. Moreover, colon adenocarcinoma, lung adenocarcinoma, and kidney renal papillary cell carcinoma were reported to be associated with high ACE2 expression [8]. Acute kidney injury plays a critical role in COVID-19 acquisition [27], and a study demonstrated that up to 25 % of critically ill patients with SARS-CoV-2 infection exhibited acute kidney injury [28]. A mechanism of acute kidney injury in patients with COVID-19 is direct renal cellular injury induced by SARS-CoV-2 due to high *ACE2* expression in the kidneys [28]. However, a specific treatment for acute kidney injury induced by COVID-19 has yet to be established. The bronchial and pulmonary epithelium and the pulmonary capillaries of the lung express ACE2 [29]. Although ACE2 can protect against lung injury due to its proinflammatory and profibrotic effects [30], Kuba et al. showed that ACE2-knockout mice could not incur SARS-CoV infection or sustain acute lung inflammation [31]. A study reported that low ACE2 expression in the lungs can protect against SARS-CoV-2 infection [32]. Thus, the critical role of ACE2 in preventing acute lung injury must be considered in the development of COVID-19 treatment strategies.

Both ACE2 and TMPRSS2 are the key proteins for SARS-CoV-2 entry [4,15–17]. Therefore, host cells with high ACE2 and TMPRSS2 expression should be considered vital for SARS-CoV-2 infection. Lukassen et al. have reported high ACE2 and TMPRSS2 expression in the lung tissue and bronchial branches [11,33]. Another study revealed that the lung,
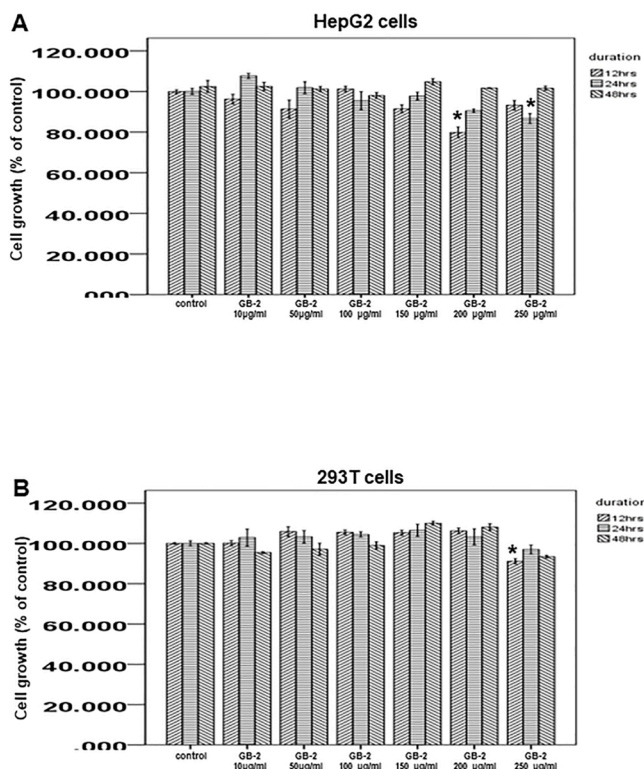
Fig. 1. Effect of GB-2 on HepG2 cells and 293 T cells. HepG2 cells (A) or 293 T cells (B) were measured by XTT assay after indicated hours of culturing in the presence of GB-2. All the results are representative of at least three independent experiments. (Error bars = mean ± S.E.M. Asterisks (*) mark samples significantly different from control group with p < 0.05).
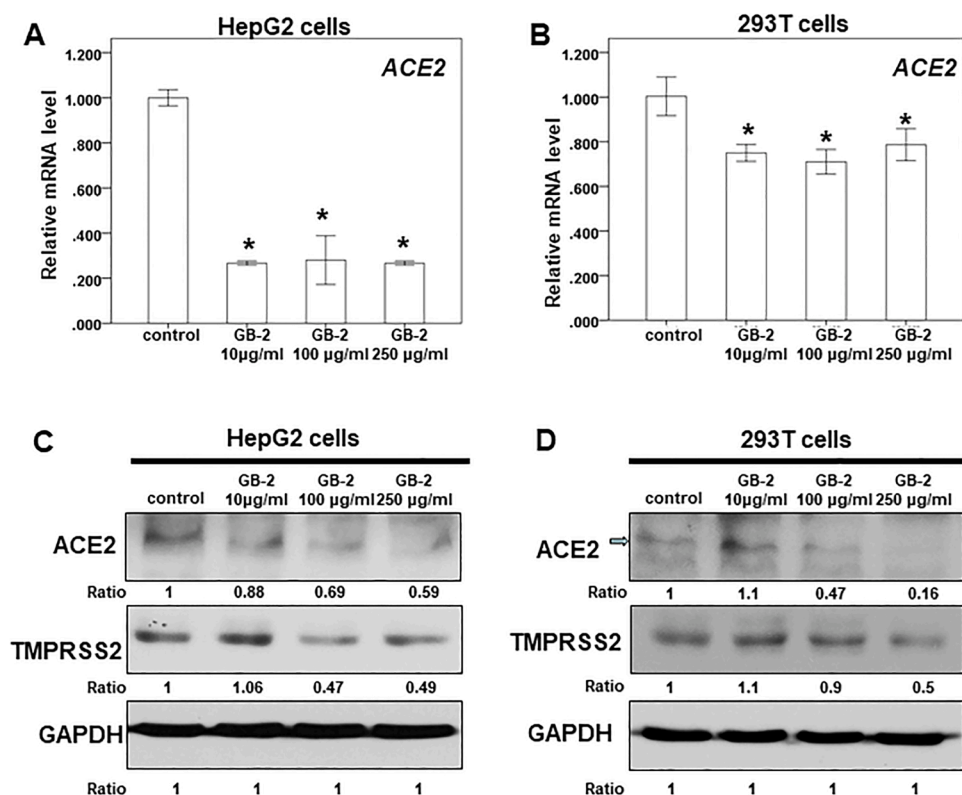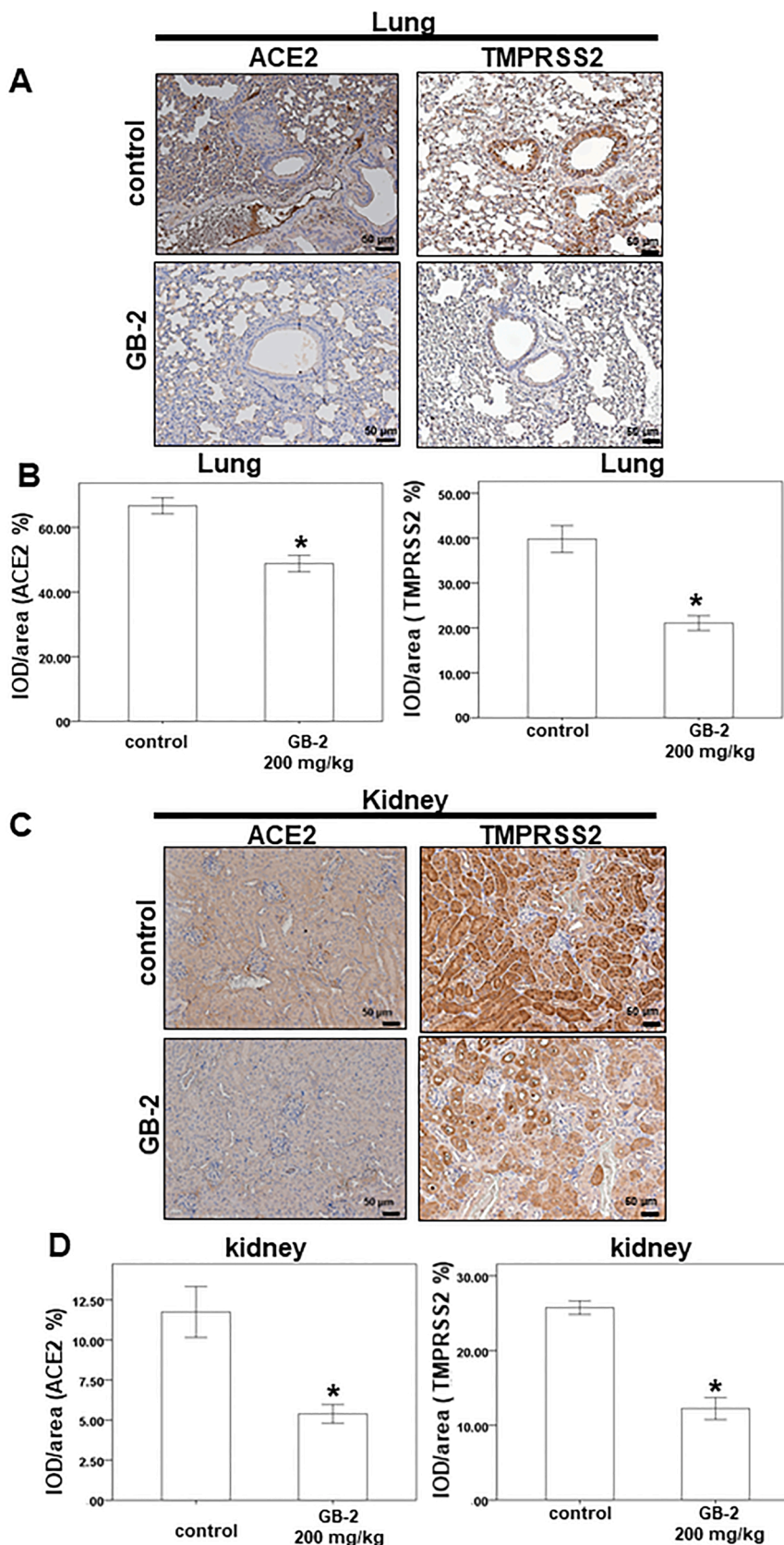
Fig. 2. Effect of GB-2 on ACE2 and TMPRSS2 expression. (A, B) Total cell extracts of HepG2 cells (A) or 293 T cells (B) were harvested from untreated cells and cells treated with GB-2 for 24 h. The protein was immunoblotted with polyclonal antibodies specific for ACE2 or TMPRSS2. GAPDH was used as an internal loading control. (C, D) Total mRNA was extracted from the HepG2 cells (C) or 293 T cells (D) after treat with GB-2 for 24 h. The coding regions of human ACE2 were used as probes for real time polymerase chain reaction analysis. All the results are representative of at least three independent experiments. (Error bars = mean ± S.E.M. Asterisks (*) mark samples significantly different from control group with $p < 0.05$).
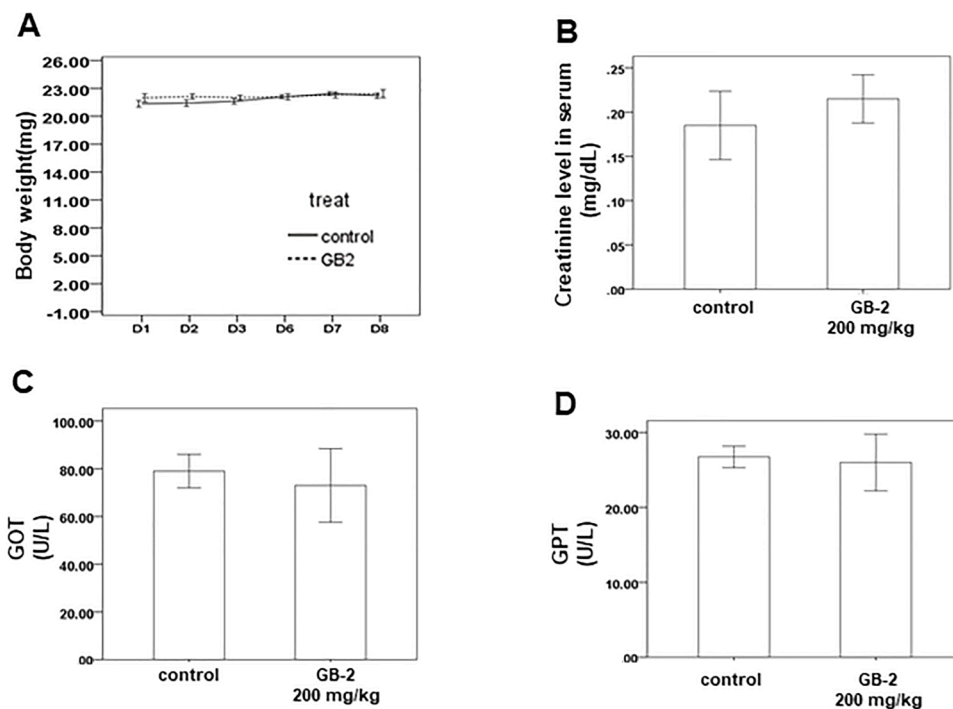

Fig. 3. In vivo effect of GB-2 on mouse model. (A) Average mice weights with vehicle/200 mg/kg/day GB-2 every day by oral administration over a time course of 1 week. (B, C, D) Creatinine (B), GOT (C) and GPT (D) levels in serum of mice after the treatment of vehicle or GB-2. All the results are representative of at least three independent experiments. (Error bars = mean ± S.E.M. Asterisks (*) mark samples significantly different from control group with $p < 0.05$).

kidney, and small intestine expressed the coding genes of both *ACE2* and *TMPRSS2* [34]. Therefore, blocking of ACE2 and TMPRSS2 activity could be an excellent anti-SARS-CoV-2 strategy. Camostat mesylate, a TMPRSS2 inhibitor, was reported to block the SARS-CoV-2 entry into host cells [4]. Furthermore, Kumar et al. reported that withanone could induce the downregulation of *TMPRSS2* mRNA in MCF-7 cells [35]. However, a potent inhibitor of both ACE2 and TMPRSS2 has not been reported until now. Our IHC data indicate high ACE2 and TMPRSS2 expression in lung and kidney tissues; this finding is consistent with those of earlier studies. Our data reveal that GB-2 could inhibit the

Fig. 4. Effect of GB-2 on ACE2 and TMPRSS2 expression on mouse model. (A, C) Representative IHC staining photomicrographs of the lung (A) and kidney (C) tissue in mice. (B, D) Quantitative results of IHC staining, which were presented as IOD/area and were proportional to the levels of ACE2 or TMPRSS2 in the lung (B) and kidney (D) tissue. (n = 5 per group, Error bars = mean ± S.E.M. Asterisks (*) mark samples significantly different from control group with p < 0.05).
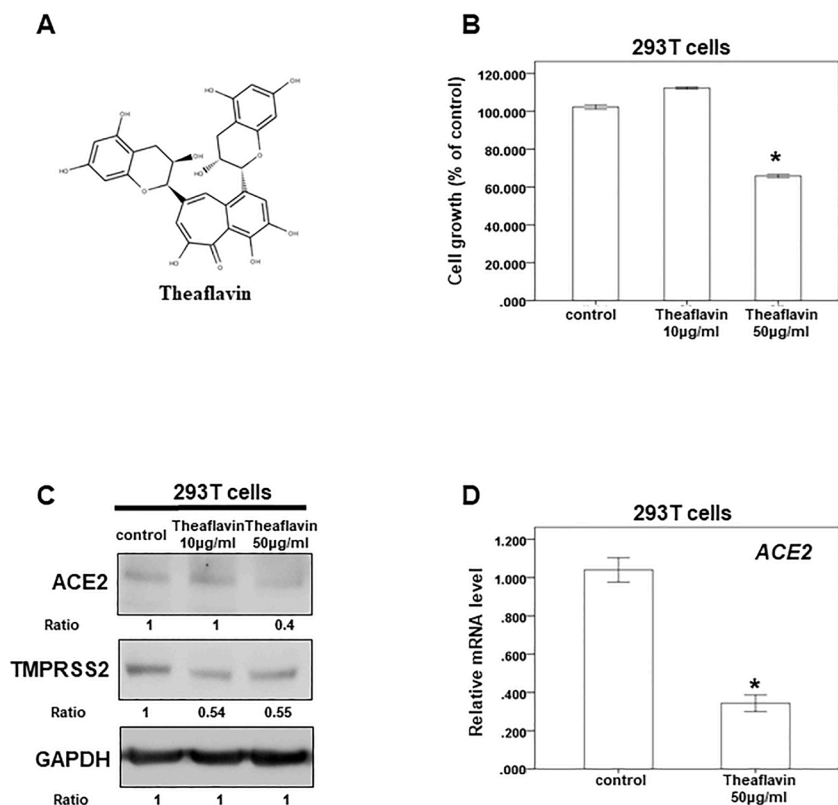
Fig. 5. Effect of theaflavin on ACE2 and TMPRSS2 expression. (A) The structure of theaflavin. (B) 293 T cells were measured by XTT assay after indicated hours of culturing in the presence of theaflavin. (C) Total cell extracts of 293 T cells were harvested from untreated cells and cells treated with theaflavin for 24 h. The protein was immunoblotted with polyclonal antibodies specific for ACE2 or TMPRSS2. GAPDH was used as an internal loading control. (D) Total mRNA was extracted from the 293 T cells after treat with theaflavin for 24 h. The coding regions of human *ACE2* were used as probes for real time polymerase chain reaction analysis. All the results are representative of at least three independent experiments. (Error bars = mean ± S.E.M. Asterisks (*) mark samples significantly different from control group with $p < 0.05$).

expression of both ACE2 and TMPRSS2 in lung and kidney tissues in animal model and could be the potential compounds for ACE2 and TMPRSS2 inhibitors in the further clinical study.

Many natural compounds have been screened as therapeutic candidates for COVID-19 treatment through in silico molecular docking method [18]. Kumar et al. reported that geranium, lemon oils, and the major compounds of lemon oils (citronellol and limonene) exhibited significant ACE2 inhibitory effects in epithelial cells [36]. Withanone, a compound from the alcoholic extract of Ashwagandha, could inhibit the mRNA expression of *TMPRSS2* in MCF-7 cells [35]. Several studies have indicated that compounds such as epigallocatechin-3-gallate and catechins extracted from *camellia sinensis* var. assamica could be potential candidates for the prevention of some viral infections, including SARS virus, Middle East Respiratory Syndrome virus, and hepatitis virus [37, 38]. However, the effects of *camellia sinensis* var. assamica and its purified compounds on SARS-CoV-2 are still unclear. In our previous study, we applied in silico molecular docking method, and our results demonstrated that the chemical structure of theaflavin resembled that of the anti-SARS-CoV-2 RNA-dependent RNA polymerase [39]. Moreover, theaflavin extracted from *camellia sinensis* var. assamica could inhibit the expression of both ACE2 and TMPRSS2 in the 293 T cell model. However, additional in vivo studies are required to ascertain the exact effect of theaflvain on SARS-CoV-2 infection. We also discovered that theaflavin could inhibit the protein expression of ACE2 and TMPRSS2 in the higher concentration (50 μg/mL). However, GB-2 could inhibit the protein expression of ACE2 and TMPRSS2 in 100 μg/mL. These results suggest that many undiscovered compounds in GB-2 may work together to inhibit the protein expression of ACE2 and TMPRSS2.

In conclusion, our results suggest that GB-2 and theaflavin, the index compound from *camellia sinensis* var. assamica, can inhibit the protein expression of ACE2 and TMPRSS2. However, the exact clinical effect remains unknown; this thus necessitates further clinical research to confirm the effects of GB-2 and theaflavin as ACE2 and TMPRSS2 inhibitors.

Authors' contributions

C.Y.W. conceived the idea and designed experiments and wrote manuscript. Y.S.L. prepared GB-2 and performed the experiments; L.H.S., Y.C.C. and H.T.L., analyzed the data. Y.H.Y. revised English writing of the manuscript. All authors reviewed and approved the final version.

Availability of data and materials

All data generated or analyzed during this study are indicated in this article (with no patient data). The datasets generated during and /or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Studies involving animals were approved by Animal Care and Use Committee (Approval number: 2,017,081,601) of Chang Gung Memory Hospital.

Consent for publication

Not applicable (not contain any individual person's data).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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