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Original Article

Tannic acid suppresses SARS-CoV-2 as a dual inhibitor of the viral main protease and the cellular TMPRSS2 protease

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Abstract: The cell surface protein TMPRSS2 (transmembrane protease serine 2) is an androgen-responsive serine protease important for prostate cancer progression and therefore an attractive therapeutic target. Besides its role in tumor biology, TMPRSS2 is also a key player in cellular entry by the SARS-CoV viruses. The COVID-19 pandemic caused by the coronavirus SARS-CoV-2 has resulted in huge losses in socio-economy, culture, and human lives for which safe and effective cures are highly demanded. The main protease (M^{pro}/$3CL^{pro}$) of SARS-CoV-2 is a critical enzyme for viral propagation in host cells and, like TMPRSS2, has been exploited for treatment of the infectious disease. Numerous natural compounds abundant in common fruits have been suggested with anti-coronavirus infection in the previous outbreaks of SARS-CoV. Here we show that screening of these compounds identified tannic acid a potent inhibitor of both SARS-CoV-2 M^{pro} and TMPRSS2. Molecular analysis demonstrated that tannic acid formed a thermodynamically stable complex with the two proteins at a K_D of 1.1 μM for M^{pro} and 1.77 μM for TMPRSS2. Tannic acid inhibited the activities of the two proteases with an IC_{50} of 13.4 μM for M^{pro} and 2.31 μM for TMPRSS2. M^{pro} protein. Consistently, functional assays using the virus particles pseudotyped (Vpp) of SARS-CoV2-S demonstrated that tannic acid suppressed viral entry into cells. Thus, our results demonstrate that tannic acid has high potential of developing anti-COVID-19 therapeutics as a potent dual inhibitor of two independent enzymes essential for SARS-CoV-2 infection.

Keywords: Tannic acid, COVID-19, SARS-CoV-2, main protease, TMPRSS2

Introduction

The cell surface protease transmembrane protease serine 2 (TMPRSS2) is an androgen-responsive serine protease important for prostate cancer progression [1-3]. Increased expression of TMPRSS2 frequently occurs in prostate cancer in response to androgen stimulation, and somatic fusion of TMPRSS2 with the ERG gene has been found to foster prostate cancer development [4]. The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been a serious global pandemic, resulting in enormous socio-economic losses. In spite of the intensive efforts invested, safe, effective, and cost-efficient drugs for COVID-19 are yet beyond the horizon. Importantly, cellular entry of SARS-CoV-2 entails the viral surface spike protein (S protein) which is activated by proteolytic processing the S protein to produce the S1 protein by TMPRSS2 [5]. The S1 protein is then recognized by and binds to the cell surface receptor ACE2 (angiotensin converting enzyme 2), a process triggers the fusion of the virus and cell membranes for virus

entry [6]. Subsequent virus maturation of the virions in host cells is through a highly coordinated proteolytic cascade of processing the viral precursor polyprotein by the main protease (M^{pro}/$3CL^{pro}$) [7-10]. The SARS-CoV-2 M^{pro} belongs to the chymotrypsin-like protease family and cleaves its substrate polypeptides at a well-defined cleavage sequence. Inhibition of M^{pro} blocks the process of viral polyproteins thus abrogates viral replication in host cells [11], making it an attractive therapeutic target of COVID-19. Numerous natural compounds have been suggested with anti-CoV activity during the previous epidemic outbreaks of SARS and MERS [12-17]. Interestingly, many of the natural compounds showing promising in suppressing viral infection are abundant in popular fruits, particularly berries, citrus, grape, and apples (see below). We therefore set out to determine the potency of these natural compounds in fruits for their inhibitory activities of SARS-CoV-2. Our results support tannic acid as a natural compound with potent anti-SARS-CoV-2 activity of targeting both SARS-CoV-2 M^{pro} and the cellular protease TMPRSS2.

Material and methods

Sample preparations

The SARS-CoV-2 M^{pro} cDNA and its fluorescent protein substrate were constructed in expression plasmids and purified as described previously [18]. Briefly, the bacterial clones harboring the IPTG-inducible expression vectors of the recombinant proteins were cultured in LB medium at 37°C until the culture reached an OD600 between 0.6-0.8. Expression of the recombinant proteins was induced with 0.5 mM IPTG or 0.2% L-rhamnose and further incubated at 20°C for 18 hr. The cells were pelleted by centrifugation and the pellets were suspended in buffer A containing 50 mM Tris pH 8.0, 0.5 M NaCl, 10% glycerol, 1 mM TCEP, 1 mM PMSF and lysed by sonication. Following centrifugation at 28,000 g, 4°C for 30 min, the supernatant was collected and separated through a Ni-column (GE Healthcare). The column was then washed by the sonication buffer supplemented by 10 mM imidazole, followed by elution with a 20-200 mM imidazole gradient in buffer A. M^{pro} recombinant protein was released by cleaving the N-terminal SUMO fusion tag with the TEV protease. M^{pro} and its substrate protein were then further purified by gel-filtration.

FRET-based enzyme activity assay of M^{pro}

Fluorescence-labeled protein substrate containing the cleavage site of M^{pro} was used for the FRET-based enzyme activity assay established in our previous study [18]. Briefly, SARS-CoV-2 M^{pro} protein (1 μM) was pre-incubated with different bioactive compounds (50 μM) for 30 min at room temperature in the assay buffer (20 mM Tris-HCl pH 7.8, 20 mM NaCl). The reaction was started by addition of 20 μM of the fluorescent protein substrate. The fluorescence signals of the cleaved products in the presence of different concentrations of tannic acid were monitored for emission at 474 nm by an excitation at 434 nm using a Synergy™ H1 hybrid multi-mode microplate reader (BioTek Instruments, Inc.). Initial velocity (V_0) was calculated in the first 15 min of the reaction by linear regression. The IC_{50} was determined using two-fold serial dilutions and analyzed by the GraphPad Prism software. All experiments were performed in triplicates. The cleavage products derived from the fluorescent protein substrates by SARS-CoV-2 M^{pro} were confirmed by separation in 12% SDS-PAGE gels and visualized by Coomassie Blue staining.

Measurement of human TMPRSS2 activity by a FRET-based enzymatic assay

Purified MBP-tagged TMPRSS2 (residues 256-492, UniProt accession: O15393) was pre-incubated in the presence or absence of tannic acid (60 μM) in the assay buffer (25 mM Tris, 150 mM NaCl) for 30 min at room temperature. After adding 20 μM of the fluorescent protein substrate the reaction was monitored using a Synergy™ H1 hybrid multi-mode microplate reader with excitation at 506 nm and emission at 536 nm. DMSO was used as the negative control.

Surface plasmon resonance (SPR) analysis

Binding kinetics of tannic acid to SARS-CoV-2 M^{pro} was assessed using a Biacore T200 (Cytiva) at 25°C with a CM5 sensor chip. The purified SARS-CoV-2 M^{pro} was dialyzed into PBS buffer pH 7.4 at 4°C for overnight before applying to the chip. The surface of the CM5 chip

was activated in EDC/NHS for 420 s. The final immobilization level of SARS-CoV-2 M^{pro} was estimated to be about 12000 response units. Tannic acid was dissolved in PBS buffer and flowed through the chip surface at a flow rate of 30 μl/min. The association and dissociation time for affinity analysis was set for 60 and 120 sec, respectively. Five dilutions of tannic acid from 25 to 1.56 μM were assessed to determine the dissociation constant (K_D) using the BIA evaluation program.

Molecular docking

The SARS CoV-2 M^{pro} structure was retrieved from Protein Data Bank (PDB code 6W63). The structures of tannic acid and M^{pro} were prepared using the BIOVIA Discovery Studio software (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2020, San Diego: Dassault Systèmes, 2016). To investigate how tannic acid interacts with SARS CoV-2 M^{pro}, LibDock was employed to generate and score the docking poses.

Vpp pseudoviral particle infection assay

The SARS-CoV2-S pseudoviral particles were purchased from the RNAi core of the Academia Sinica, Taiwan. The human embryonic kidney cell line 293T stably expressing recombinant human ACE2 (293/hACE2) were maintained in Dulbecco's MEM containing 10% fetal bovine serum, 100 unit penicillin, and 100 μg streptomycin. The 293/hACE2 cells were seeded into 96-well plates and pre-treated with different doses of tannic acid for 1 h, then inoculated with 100 μl of normal media containing the pseudovirions (MOI = 0.28). After overnight incubation, cells were fed with fresh media. At about 40 h post-inoculation, cells were lysed with 100 μl medium containing 50% Steady-Glo (Promega) at room temperature for 5 min. The transduction efficiency was measured by quantification of the luciferase activity using an ELISA reader. The cytotoxicity of tannic acid to 293T/hACE2 cells was assessed by using the Cell Counting Kit-8 kit (ab228554, Abcam) following the manufacture's protocol. Briefly, 5×10^4 cells per well in 96 well plates were seeded in 100 μl of media and cultured for about 24 h, followed by treatment with different doses of tannic acid in 100 μl DMEM/high glucose medium containing 5% FBS for 24 h. CCK-8 solution (10 μl) was added and incubated for 3 h. The OD values (460 nm) were measured to represent cell proliferation ability. All experiments were performed in triplicates, and repeated three times.

The African green monkey kidney cells Vero E6 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1x GlutaMAX, and 1% penicillin/streptomycin. Cells were seeded into 96-well plates and pre-treated with different doses of tannic acid for 1 hr, then inoculated with 50 ml pseudovirions (MOI = 0.28). After incubation for 24 h, cell viability was confirmed by the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories). Each sample was then mixed with an equal volume of luciferase substrate of the Bright-Glo Luciferase Assay System (Promega), and luminescence was measured immediately by the GloMax Navigator System (Promega). Viability-normalized relative light unit (RLU) was set as 100% for the control group, and the relative infection efficiencies of the treated groups were calculated. The cytotoxicity of tannic acid to Vero E6 was assessed by seeding 2×10^4 cells per well in 24 well plates in 500 μl media and cultured for about 24 h, followed by treatment with different doses of tannic acid in 500 μl DMEM/high glucose medium containing 2% FBS for 48 h. The media was then removed and viable cells were detected with MTT assay which was read by absorbance at 570 nm by an ELISA reader. All experiments were performed in triplicates, and repeated three times.

Results

Six previously identified bioactive natural compounds that were effective against CoV, namely catechin [12], kaempferol [15], quercetin [15], proanthocyanidins [16], resveratrol [14], and tannic acid [12], were assessed for their ability to inhibit the enzymatic activity of SARS-CoV-2 M^{pro} using a fluorescence resonance energy transfer (FRET)-based assay to measure the enzymatic activity of M^{pro} [19, 20] (**Table 1**). Among the six compounds tested, only tannic acid showed up with significant activity of inhibiting up to 90% of the enzymatic activity of SARS-CoV-2 M^{pro} at a concentration of 50 μM (**Figure 1A**; data not shown). The cleavage was verified with SDS polyacrylamide

Table 1. List of dietary sources of fruit bioactive compounds shown to be active in suppression SARS-CoV infection

	Source fruits	Proposed targets	Ref.
(+)-Catechin	apples, blueberries, gooseberries, grape seeds, kiwi, strawberries	$3CL^{pro}$	[12]
Quercetin	apples, citrus fruits, grapes, red raspberry, nectarine	PL^{pro}	[15]
Tannic acid	berries, grapes, persimmons, pomegranate	$3CL^{pro}$	[12]
Proanthocyanidins	blueberries, cranberries, black currant, plums, grapes	infection	[16]
Resveratrol	blueberries, bilberries, cranberries, grapes	apoptosis	[14]
Kaempferol	apples, grapes, tomatoes, peaches, blackberries, raspberries	PL^{pro}	[15]

$3CL^{pro}$, 3-chemotrypsin-like protease; PL^{pro}, papain-like protease.

gel electrophoresis (SDA-PAGE) to visualize the produced peptides (**Figure 1B**). A dose-response analysis estimated a half-maximal inhibitory concentration (IC_{50}) of tannic acid at 1.3×10^{-5} M in SARS-CoV-2 M^{pro} inhibition (**Figure 1C**). These results raise the question of whether tannic acid directly interacts with SARS-CoV-2 M^{pro} to inhibit its activity. To determine the dose-dependent affinity of tannic acid to SARS-CoV-2 M^{pro}, 5 dilutions of tannic acid from 25 μM to 1.56 μM were tested. The dissociation constant (K_D) for tannic acid binding to SARS-CoV-2 M^{pro} was determined to be 1.1×10^{-6} M from the association and dissociation curves of the sensorgrams, using the Biacore evaluation program (**Figure 2A**). To further understand the mechanism of M^{pro} inhibition by tannic acid, the interaction between them was modeled by docking analysis. The crystal structure of SARS-CoV-2 M^{pro} in its ligand-free state has been resolved in previous studies [11, 18, 21]. Each SARS-CoV-2 M^{pro} molecule is composed of three domains. Domain I (residues 10 to 100) and II (residues 101 to 182) have a chymotrypsin-like, two-β-barrel fold commonly found in the structure of M^{pro} of other human and animal coronaviruses, such as PEDV, MERS-CoV, and SARS-CoV [9, 22, 23]. Domain III (residues 200 to 304) of SARS-CoV-2 M^{pro} includes five α-helices that form a globular structure. Given that the two viruses share 96.08% sequence identity, it is not surprising that the structure of the SARS-CoV-2 M^{pro} is highly similar to that of the SARS-CoV M^{pro} [21]. The domains I and II occupy the substrate-binding pockets and are evolutionarily highly conserved. In the helical domain III, on the other hand, the surface loops of CoV M^{pro}s harbor regions of intrinsic variation [11]. The structure of tannic acid is characterized by a central glucose molecule esterified at the five hydroxyl groups with one or more gallic molecules [24]. Docking analysis showed that tannic acid occupies the pocket defined by binding with the key residue Cys145 via the pi-sulfur and hydrogen bond with 4.69 Å and 2.62 Å, respectively, as well as Asn142 and Met165 via the hydrogen bond and pi-alkyl interactions (**Figure 2B**).

The potential activity of tannic acid in SARS-CoV-2 infection was assessed by a SARS-CoV-2 pseudovirus assay in which the human baby kidney 293T cells overexpressing the S protein receptor ACE2 were used as the target cells in a pseudoviral particle infection assay. Intriguingly, tannic acid treatment significantly inhibited the entry of SARS-CoV-2 pseudovirus in a dose-dependent manner in the absence of significant cytotoxicity (**Figure 3A** and **3B**). Similar effect of entry inhibition by tannic acid was also observed in Vero E6 cells (**Figure 3C** and **3D**). This result suggests that, besides the intracellular proteolytic enzyme M^{pro}, tannic acid can also target the mechanisms governing virus entry. Indeed, *in vitro* enzymatic assay of TMPRSS2 showed that treatment with tannic acid inhibited the protease activity of TMPRSS2 (**Figure 4A**). To further test whether tannic acid is a direct inhibitor of TMPRSS2, the dose-dependent affinity of tannic acid to human TMPRSS2 was determined by measuring 5 dilutions of tannic acid from 25 μM to 1.56 μM. Analysis of the association and dissociation curves of the sensorgrams showed a dissociation constant (K_D) of 1.77×10^{-6} M for tannic acid binding to TMPRSS2 using the Biacore evaluation program (**Figure 4B**). Thus, these results together demonstrate that tannic acid is an inhibitor with dual inhibitory functions of blocking both viral and cellular serine proteases critical for viral infection.

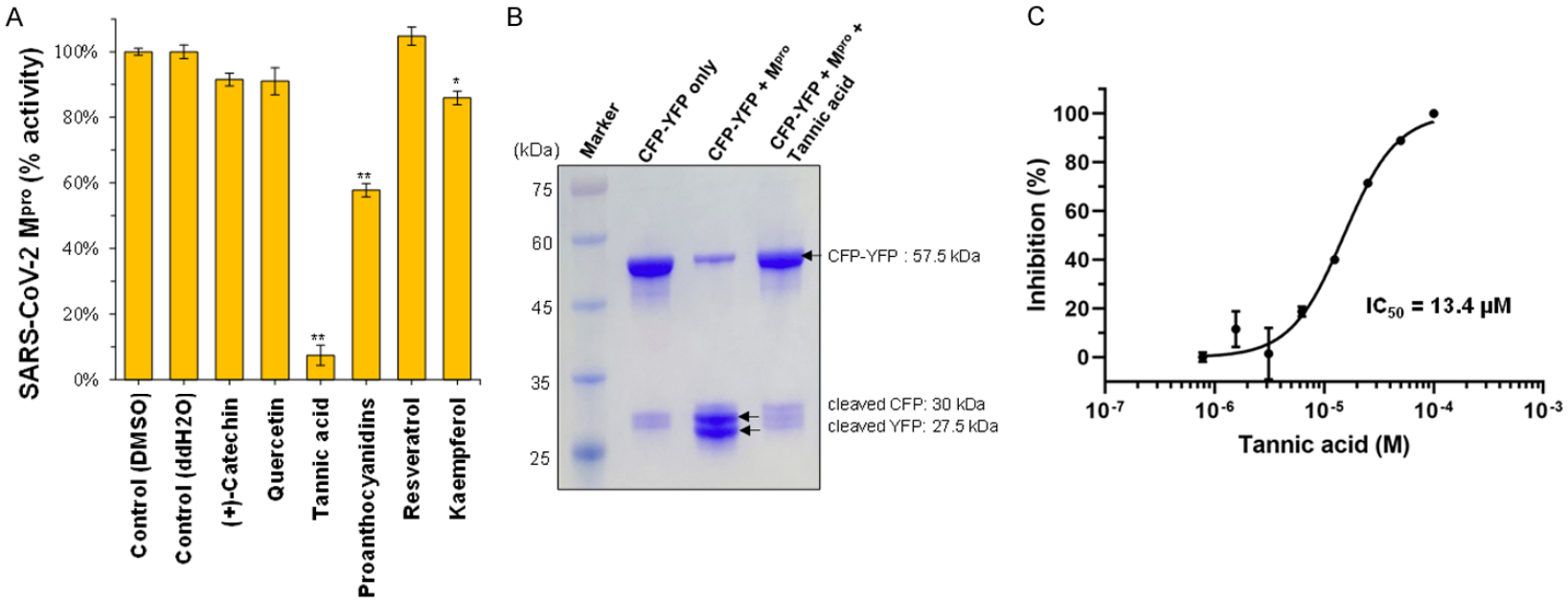

Figure 1. Tannic acid is a promising direct inhibitor of M^{pro}. A. Screening of previously identified bioactive compounds in fruits that were effective in inhibiting M^{pro} of SARS-CoV-2. 50 μM bioactive compounds were pre-incubated with 1 μM of SARS-CoV-2 M^{pro} for 30 min at room temperature. The fluorescent substrates (20 μM) were then added to initiate the reaction. The first 15 min of the reaction was used to calculate initial velocity (V_0) and then normalized to control. Tannic acid was dissolved in water, other compounds were dissolved in DMSO. Both water and DMSO were used as negative control as indicated. Data are shown as mean ± SEM from experiments performed in triplicate. Statistical significance was calculated using Student *t*-test, *P < 0.05, **P < 0.01. B. Inhibition of peptide cleavage activity of SARS-CoV-2 M^{pro} by tannic acid. M, marker. Lane 1, the fluorescent substrate (CFP-TSAVLQSGFRKM-YFP, 57.5 kDa) only. Lane 2, SARS-CoV-2 M^{pro} was incubated with the fluorescent substrate at 30°C for 1 hour. Two separate bands correspond to CFP-TSAVLQ (30 kDa) and SGFRKM-YFP (27.5 kDa) were detected. Lane 3, SARS-CoV-2 M^{pro} was incubated with the fluorescent substrate plus 50 μM tannic acid at 30°C for 1 hour. The fluorescent substrate remains uncleaved. C. Dose-effect course of tannic acid in inhibiting SARS-CoV-2 M^{pro}. The IC_{50} was calculated by plotting the initial velocity against different concentrations of tannic acid as shown by a dose-response curve generated with the Prism 8 software.

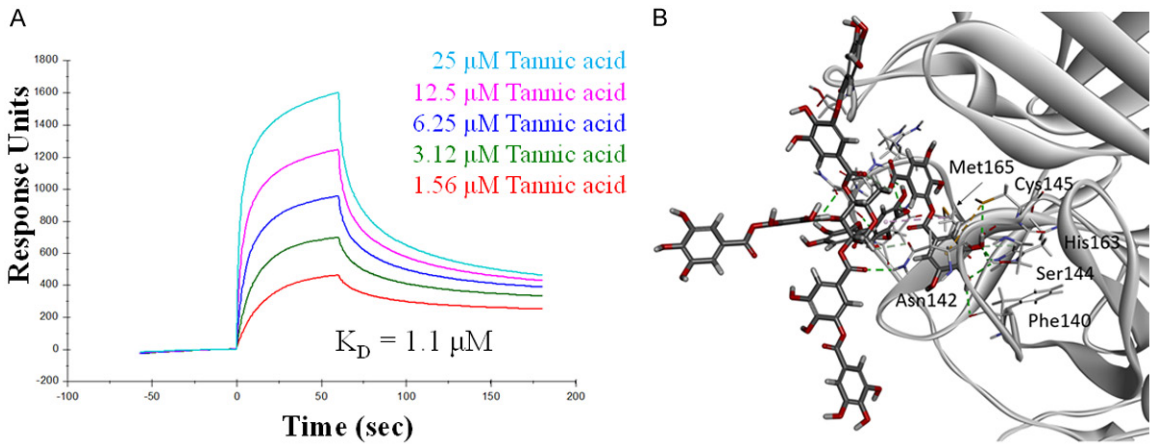

Figure 2. Association of tannic acid with SARS-CoV-2 M^{pro}. A. Binding kinetics of tannic acid to SARS-CoV-2 M^{pro}. Surface plasmon resonance analysis is performed using a BiacoreT200 with sensor chip CM5 at 25 °C. Tannic acid was dissolved in PBS buffer pH 7.4 and flowed through the chip surface at a flow rate of 30 μl/min. B. Molecular docking of tannic acid to SARS-CoV-2 M^{pro}. Tannic acid and the interacting side-chain residues are shown in sticks. Hydrogen bonds are colored in green. Pi-sulfur bond is colored in yellow. Pi-alkyl bond is colored in light pink.

Discussion

Currently there are no clinically proven drugs specifically targeting SARS-CoV-2 except for remdesivir which has been approved by US FDA for emergency use. However, a randomized, double-blinded, placebo-controlled clinical studies have shown that treatment by remdesivir did not result in difference in time to clinical improvement (hazard ratio 1.23 [95% CI 0.87-1.75]) [25]. On the other hand, among the patients with symptom duration of 10 days or less, individuals received remdesivir did have a trend to achieve clinical improvement faster than those received placebo. The difference compared with a placebo, however, is not statistically significant (hazard ratio 1.52 [0.95-2.43]) [25]. Like remdesivir, the six compounds selected for this study were also based on the prior applications in coping with viral outbreaks, particularly for CoVs.

Tannic acid belongs to the tannin family. Tannins are the major components affecting the richness of texture of red wine. It is estimated that the enological concentrations of tannins in red wines range from 5~100 μM depending on the variety. Tannic acid is a water-soluble polyphenol frequently present in herbaceous and woody plants, legumes, sorghum, as well as the commonly consumed fruits such as raspberries, bananas, and persimmons [24]. The polyphenol compounds are well known for their redox activity as antioxidants and radical scavengers [26, 27]. To this regards, a cohort of recent studies have demonstrated the activity of tannic acid in suppressing multiple biological functions in cancer cells from energy metabolism, cell proliferation, invasion, metastasis, to anti-inflammation, establishing tannic acid as a chemopreventing and chemosensitizing anticancer agent [28, 29]. Further work is required to determine whether tannic acid inhibits cancer progression through targeting the TMPRSS2 membrane protease. Overall our results highlight the potential of mining natural sources as reservoirs of safe and effective remedies to curb the COVID-19 pandemic besides neoplastic diseases.

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Disclosure of conflict of interest

A provisional patent application has been submitted to the United States Patent and Trademark Office (63/106,938). Mien-Chie Hung, Yeh Chen, and Shao-Chun Wang are co-inventors.

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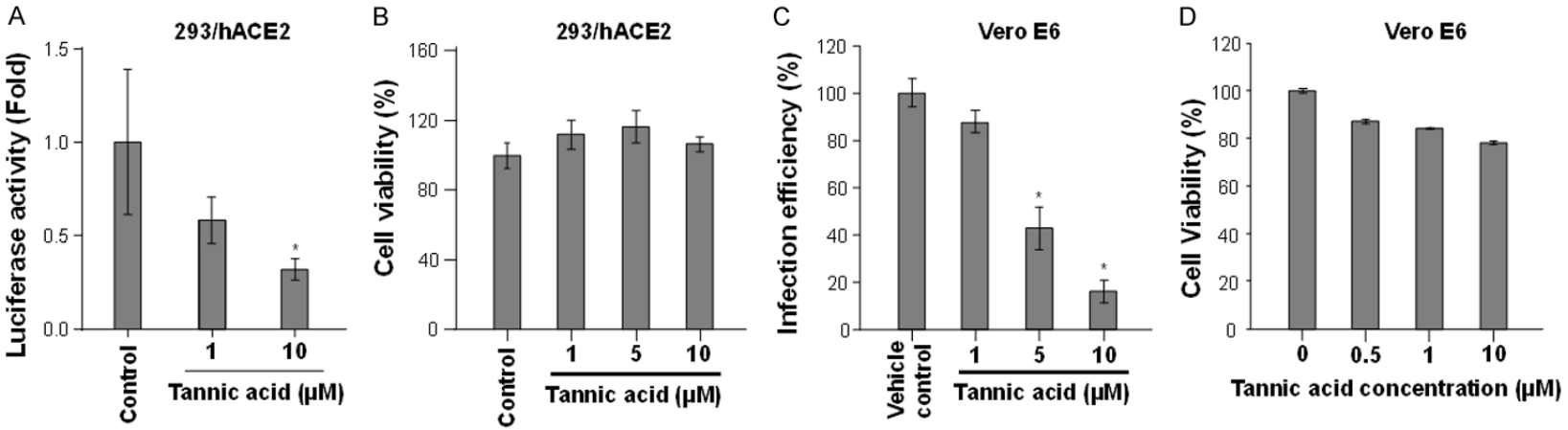

Figure 3. Tannic acid blocked cell entry of the SARS-CoV-2 pseudovirus. A. 293T/hACE2 cells were pre-incubated with the indicated doses of tannic acid and cell entry of the SARS-CoV2-S pseudoviral particles was assessed by a luciferase reporter assay. B. The cytotoxicity of 293/hACE2 cells treated with the indicated concentrations of tannic acid for 24 h as measured by cell viability. C. Vero E6 cells were pre-incubated with the indicated doses of tannic acid and cell entry of the SARS-CoV2-S pseudoviral particles was assessed by a luciferase reporter assay. D. The cytotoxicity of Vero E6 cells treated with the indicated concentrations of tannic acid for 48 h as measured by cell viability. The detail procedures of measuring viral entry and cytotoxicity are described in Materials and Methods. Bars, standard deviations; *, P < 0.05 as determined by Student's *t* test.

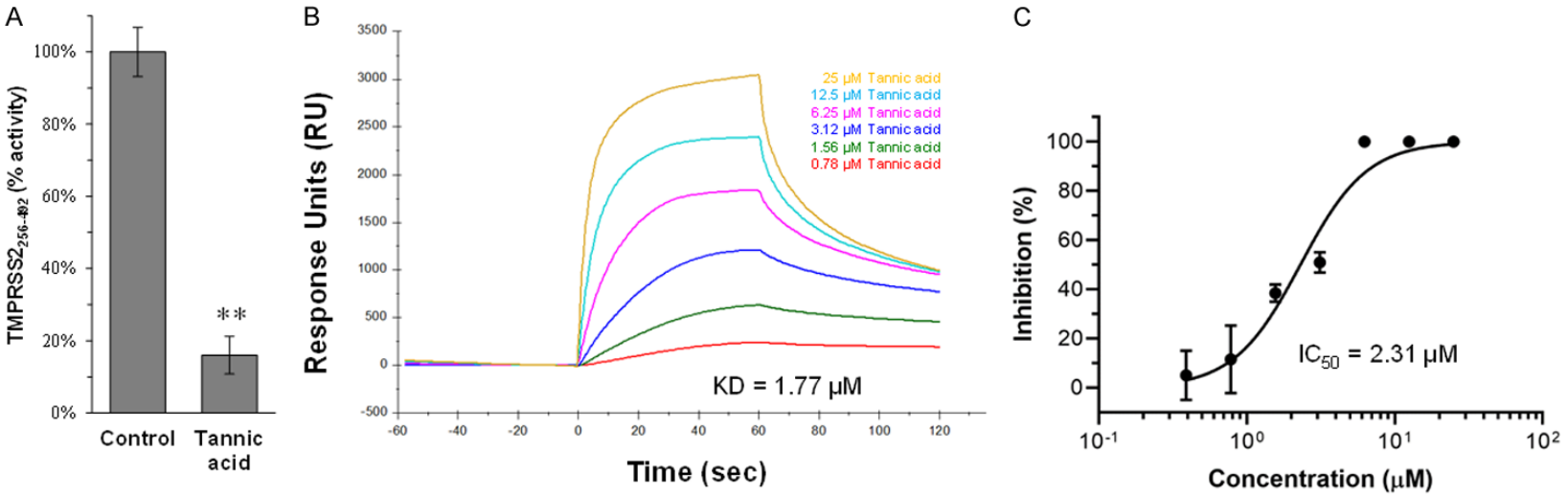

Figure 4. Tannic acid is an inhibitor of TMPRSS2. A. Recombinant TMPRSS2 was mock treated or treated with tannic acid (60 μM) and the enzymatic activity was determined. **, $P < 0.01$. B. Binding kinetics of tannic acid to $TMPRSS2_{256-492}$. SPR analysis is performed using a BiacoreT200 with sensor chip CM5 at 25°C. Tannic acid was dissolved in PBS buffer pH 7.4 and flowed through the chip surface at a flow rate of 30 μl/min. Six dilutions of tannic acid from 25 to 0.78 μM were analyzed to determine the binding affinity to serine protease domain of TMPRSS2. The experiments were repeated. C. Tannic acid inhibits the protease activity of $TMPRSS2_{256-492}$. IC_{50} (2.31 μM) of tannic acid was determined based on 7 dilutions from 25 to 0.39 μM and analyzed by GraphPad Prism software.

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