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Discovery of *Camellia sinensis* catechins as SARS-CoV-2 3CL protease inhibitors through molecular docking, intra and extra cellular assays

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**ABSTRACT**

**Background and purpose:** Previous studies suggest that major *Camellia sinensis* (tea) catechins can inhibit 3-chymotrypsin-like cysteine protease (3CLpro), inspiring us to study 3CLpro inhibition of the recently discovered catechins from tea by our group.

**Methods:** Autodock was used to dock 3CLpro and 16 tea catechins. Further, a 3CL pro activity detection system was used to test their intra and extra cellular 3CLpro inhibitory activity. Surface plasmon resonance (SPR) was used to analyze the dissociation constant (Kd) between the catechins and 3CLpro.

**Results:** Docking data suggested that 3CLpro interacted with the selected 16 catechins with low binding energy through the key amino acid residues Thr24, Thr26, Asn142, Gly143, His163, and Gln189. The selected catechins other than zijuanin D (3) and (-)-8-(5′R)-N-ethyl-2-pyrrolidinone-3-O-cinnamoyl epicatechin (11) can inhibit 3CLpro intracellularly. The extracellular 3CLpro IC50 values of (-)-epicatechin 3-O-caffeate (EC-C, 1), zijuanin C (2), etc-pyrrolidinone A and D (6), etc-pyrrolidinone A (9), etc-pyrrolidinone C and D (6), etc-pyrrolidinone A (9), etc-pyrrolidinone C gallate (GCG), and (-)-epicatechin gallate (ECG) are 1.58 ± 0.21, 41.2 ± 3.56, 0.90 ± 0.03, 46.71 ± 10.50, 3.38 ± 0.48, and 71.78 ± 8.36 µM, respectively. The Kd values of 1, 6, and GCG are 4.29, 3.46, and 3.36 µM, respectively.

**Conclusion:** Together, EC-C (1), etc-pyrrolidinone C and D (6), and GCG are strong 3CLpro inhibitors. Our results suggest that structural modification of catechins could be conducted by esterifying the 3-OH as well as changing the configuration of C-3, C-3′ or C-5′ to discover strong SARS-CoV-2 inhibitors.

**Introduction**

The novel coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) has spread rapidly around the world and has become a global health emergency (Li et al., 2020). SARS-CoV-2 is an enveloped single-stranded RNA virus (Oberfeld et al., 2020). 3-Chymotrypsin-like cysteine protease (3CLpro) or main protease is one of the most important proteins of the virus, which has already been identified as an important pharmacological target in the severe acute respiratory coronavirus syndrome (SARS-CoV) and Middle East respiratory syndrome virus (MERS) viruses. This protein triggers the production of a whole series of enzymes necessary for the virus to carry out its replicating and infectious activities. (Grottesi et al., 2020). Meanwhile, since a protease homologous to 3CLpro is not present in the human body, 3CLpro becomes an ideal anti-coronavirus target, which is responsible for processing polyproteins of nidoviruses and picornaviruses (Kim et al., 2016).

*Camellia sinensis* (L.) Kuntze (Theaceae) (common name ‘tea’) is normally classified into six major types (green tea, white tea, yellow tea, oolong tea, black tea, and dark tea) according to the processing manufacture, and is popularly consumed around the world (Ke et al., 2019). Green tea, black tea, and oolong tea were reported to inhibit the contagious virus SARS-CoV-2 dose-dependently by in vitro cell assays (Nishimura et al., 2021). Green tea can inhibit SARS-CoV-2 3CLpro with...
a half-maximal inhibitory concentration (IC$_{50}$) at 8.9 ± 0.5 µg/ml (Upadhyay et al., 2020). We used SARS-CoV-2 3CLpro for screening potential agents against the current fast epidemic since this protein has been used previously selected to screen anti-SARS-CoV-2 agents in silico and in vitro (Jang et al., 2021; Zhu and Xie, 2020). Common catechin monomers have high micromolar IC$_{50}$ values while with in vivo concentrations of less than 1 µM, searching for stronger catechins with small IC$_{50}$ values is highly urgent and realizable for their utilization.

Researchers have found that introducing new chemical groups into the structure of catechins can significantly improve their stability and bioavailability, and specifically enhance their pharmacological effects (Liu et al., 2021; Xiao et al., 2013). Moreover, the effects of substitution at different positions are different. Thus, we selected 16 catechins (compounds 1-12), which had recently been isolated from tea by our group including the major tea catechins (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (+)-catechin gallate (CG), and (+)-gallocatechin gallate (GCG) to test their 3CLpro inhibition activity. These catechins have previously demonstrated different biological activities. (-)-Epicatechin 3-O-cafeoate (1, EC-C) can inhibit acetylcholinesterase activity (Wang et al., 2017), form complex with iron and neutrophil gelatinase-associated lipocalin, and protect against β-amyloid (Aβ) induced neurotoxicity in SH-SYSY cells (Zhang et al., 2018). Zijuanin C (2) and zijuanin D (3) are catechin esters with impressive activity in protecting SH-SYSY cells against H$_2$O$_2$-induced damage (Ke et al., 2019). Compounds 4-9 are ester-type flavoalkaloids isolated from white tea (Bai-Mudan) and Chinese ancient cultivated tea (Xi-Gui), which can inhibit the accumulation of advanced glycation end products and cell senescence (Cheng et al., 2018; Li et al., 2018). Compounds 10-12 are four flavoalkaloid cinnamoyl esters with strong acetylcholinesterase inhibitory effects (Gaur et al., 2020).

Luciferase (Luc) refers to a class of enzymes that catalyze specific luciferin substrates to produce bioluminescence. Several luciferases require no post-translational processing for enzymatic activity and show a linear relationship between concentration and their resulting bioluminescence (Wet et al., 1986). These properties render them excellent genetic reporters. Luc-fused proteins can be easily quantified by measuring their catalyzed bioluminescence with a luminometer, providing the detection sensitivity up to femtogram level (Williams et al., 1989). Luc biosensor system has the advantages of high sensitivity, ease of use, and applicability, which makes it a powerful tool for studying viral protease proteolysis events in living cells and achieving high-throughput screening of antiviral agents. Therefore, in this study, a cell-level screening model for the 3CLpro inhibitor of SARS-CoV-2 was established using a 3CLpro activity detection system. The intracellular detection system contains the following plasmids: a plasmid expressing 3CLpro substrate which carries renilla luc; a plasmid expressing 3CLpro; and a luc plasmid. A luc gene and a protein aggregation group gene are fused and expressed, and a 3CLpro enzyme peptide segment is arranged between the two genes. If the 3CLpro is cut at the peptide segment, the luc and the protein aggregation group are separated, leading to an active luc protein and a generated chemiluminescent light signal; conversely, when the substrate is not cut by 3CLpro, the substrate is quenched near two fluorescent groups at both ends, one of which is a quenching group. When the substrate is not cut by 3CLpro, the substrate is quenched near fluorescence, and there is no fluorescence signal. When the substrate is separated by 3CLpro, a fluorescence signal will be generated. Finally, the binding K$_D$ values of selected catechins and 3CLpro were determined by surface plasmon resonance (SPR) technology.
Materials and methods

Materials

Analytical grade reagents used for extraction and isolation were purchased from Chengdu Kelong Chemical Reagent Co., Ltd (Chengdu, China). CM7 sensor chip, 10 × PBS-P buffer (containing 0.2 M phosphate buffer, 27 mM and 1.37 M NaCl, 0.5% Surfactant P20, pH adjusted to yield pH 7.4 when diluted 10 × and supplemented with 2% DMSO), sodium acetate pH 4.0, 4.5, 5.0, 5.5 and amino coupling kit were purchased from Cytiva (Uppsala, Sweden). Dimethyl sulfoxide (DMSO) was purchased from Gentheim (Beijing, China). 293T/17 cells (CBP6044) were purchased from Cytiva (Uppsala, Sweden). Dimethyl sulfoxide (DMSO) was purchased from PreceDo Pharmaceuticals Co. Ltd (Hefei, China). 3CLpro activity detection system was purchased from Vazyme (Nanjing, China). DMEM (GentaHold (Beijing, China). Dimethyl sulfoxide (DMSO) was purchased from cobioer biosciences Co. Ltd (Nanjing, China).

%inhibition = 100 – (RLU compound / Flu compound) × 100%

Extracellular 3CLpro inhibition activity

We made a 100 × compound solution in DMSO : H₂O (1:1), added 4 µl 100 × compounds to 36 µl buffer, and diluted the compounds with growth medium to 10 × final concentration. We mixed 1 µl 3CLpro, 2 µl compounds, and 15 µl buffer (50 mM Tris,1 mM EDTA) as the reaction system. The concentration of 3CLpro was 1400 µg/ml, and the compound concentrations were 0.015, 0.045, 0.14, 0.41, 1.24, 3.70, 11.10, 33.33, and 100 µM. Inhibition (%) was calculated relative to vehicle (DMSO) treated control wells using the following formula, and data were analyzed using Graphpad 7.0 software (San Diego, CA, USA) was used to analyze the data and fit it to a 4-parameter equation to generate a concentration-response curve.

%inhibition = RLU compound/RLU DMSO control × 100%

Immobilization of 3CLpro on the chip surface

PBS-P buffer was selected as the coupling buffer, and 10 × PBS-P (pH 7.4) was diluted 10 times to prepare a 200 ml running buffer. 3CLpro was diluted to 10 µg/ml, with sodium acetate at pH 5.50, 5.00, 4.50, and 4.00, respectively, and 100 µl was prepared for each pH. Through a pre-enrichment experiment, pH 5.00 was determined as the best coupling condition. Therefore, the ligand solution was diluted to 10 µg/ml, with sodium acetate and pH 5.00. The formal coupling operation was carried out with 200 µl.

First, we injected a freshly prepared mixture of N-hydroxysuccinimide and N-ethyl-N‘-(dimethylaminopropyl) carbodiimide (1:1 v/v) at a flow rate of 10 µl/min for 420 s to activate the CM7 chip. Next, we injected the 3CLpro, at a concentration of 10 µg/ml in immobilization buffer (10 mM sodium acetate at pH 5.00) into the sample channel and allowed the 3CLpro to react the CM7 chip for 7 min at a flow rate of 10 µl/min, resulting in 3CLpro immobilized densities averaging 4000 RU. At last, we injected a solution of ethanolamine hydrochloride at pH 4.50 at a flow rate of 10 µl/min, and block the remaining carboxyl groups.

For the interaction experiments, the solutions of compounds were prepared in 1 × PBS-P buffer used in the interaction between protein and
catechins. We analyzed a range of concentrations (0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 μM) to obtain the sensorgrams of the interactions between 3CLpro and the catechins. The catechins were injected onto the 3CLpro immobilized chip for 180 s at a flow rate of 20 μl/min and 1 × PBS-P buffer was injected for 160 s at a flow rate of 20 μl/min to regenerate the chip surface at the end of each experiment. Sensorgrams were processed by using automatic correction for nonspecific bulk refractive index effects. The equilibrium dissociation constants (K_i) were determined by fitting the SPR data using non-linear fitting of the SPR signal at the steady-state with a Langmuir linear fitting of the SPR signal at the steady-state with a Langmuir

To understand the inhibition of these docking promising catechins against 3CLpro, we used 16 tea catechins obtained from our group. Their purity was detected by HPLC (Fig. S1-2) (Cheng et al., 2018; Gaur et al., 2020; Ke et al., 2019; Li et al., 2018; Wang et al., 2021) including the known major tea catechins EGC, EGCG, CG and GCG to perform intracellular inhibition assay. Ebselen (1-[2-phenyl-1, 2-benzoisoselenazol-3 (2H-one)] was used as the positive control (IC_50 = 69.70 ± 0.28 nM). Except for compounds zijuanin D (3) and (-)-8-(5’R)-N-ethyl-2-pyrrolidinone-3-O-cinnamoyl(epicatechin (11), other catechins showed dose-dependent inhibition against 3CLpro (Fig. 3, Fig. S3-S5). When the concentration is 100 μM, ECC (1), etc-pyrrolidinone C and D (6), EGC, and GCG have stronger intracellular 3CLpro inhibition (with the ratios at 93.55 ± 0.06, 93.66 ± 0.14, 79.69 ± 1.70, 93.56 ± 0.04 %, respectively) than others.

### Extracellular 3CLpro inhibition

The IC_50 values for EC-C (1), zijuanin C (2), etc-pyrrolidinone C and D (6), etc-pyrrolidinone F (8), GCG, and ECC were 1.58 ± 0.21, 41.20 ± 3.56, 0.90 ± 0.03, 46.71 ± 10.50, 3.38 ± 0.48, and 71.78 ± 8.36 μM (Fig. 4, Fig. S6-8), respectively. IC_50 values of other compounds are higher than 100 μM. The IC_50 of the positive drug ebselen is 40.00 ± 0.40 nM. Three compounds (1, 6, and GCG) have IC_50 values less than 10 μM (Fig. 4). This extracellular result is consistent with that of the intracellular one.

### Surface plasmon resonance (SPR) analysis

To obtain the dissociation constants between 3CLpro and catechins, SPR data have been analyzed by fitting the SPR sensorgrams using non-linear fitting of the SPR signal at the steady-state using a Langmuir
binding isotherm model. Five concentration gradients of each analyte were plotted with the response value at equilibrium. Rmax is the maximum response value. Offset is the minimum response value. The fitting efficiency is calculated by the Chi$^2$ value. **Table S2** shows that EC-C (1), etc-pyrrolidinone C and D (6), and GCG have low K$_D$ values (4.29, 3.46, and 3.63 µM, respectively) (Fig. 5), indicating that they all have low binding affinities for the protease.

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**Fig. 3.** Intracellular inhibition of (-)-epicatechin 3-O-caffeate (1), etc-pyrrolidinone C and D (6), (-)-epicatechin gallate (ECG), and (+)-gallocatechin gallate (GCG) against SARS-CoV-2 3CL protease activity. Data are means ± SD from three experiments. Ebselen [(2-phenyl-1, 2-benzoisoselenazol-3(2H)-one)] was used as the positive control (IC$_{50}$ = 69.70 ± 0.28 nM), Z-factor = 0.83.

**Fig. 4.** Extracellular Inhibition of ebselen, (-)-epicatechin 3-O-caffeate (1), etc-pyrrolidinone C and D (6), and (+)-gallocatechin gallate (GCG) against SARS-CoV-2 3CL protease activity. Data are means ± SD from three experiments. The IC$_{50}$ of the positive drug ebselen is 40.00 ± 0.40 nM.
strong 3CLpro binding affinity. EGCG was used as the positive control with $K_D$ value at 4.11 μM (Fig. 5). The lower calculated Chi$^2$ value indicates a good accuracy of the fitting. This means that these catechins can bind 3CLpro tightly.

**Discussion**

Searching for leading natural products from functional food is always a safe and attractive approach for the prevention, alleviation, and treatment of human diseases. Tea, as a traditional safe drink containing amounts of polyphenols, has various antiviral activities (Gaur and Bao, 2021). Specifically, green tea has anti-SARS-CoV-2 and 3CLpro inhibition effects (Upadhyay et al., 2020), suggesting that green tea could be an effective resource for searching and subsequent designing 3CLpro inhibitors against the contagious virus.

We selected 16 catechins for molecular docking with 3CLpro (Table S1). The molecular docking results suggest that these tea catechins could be potential 3CLpro inhibitors. However, the binding sites of catechins at 3CLpro are different although they are in the same pocket (Fig. 2). EC-C (1) interacts with the protein through ten hydron bonds at Thr26, Gly143, Phe140, His172, Glu166, Gln189, and Ser46. 3CLpro binds etc-pyrrolidinone C and D (6) through six hydrogen bonds at Leu4, Thr24, Thr26, Leu141, Asn142. ECG interacts with 3CLpro through five hydrogen bonds at Thr25, Asn142, Gly143, His163 while GCG interacts with it through ten hydrogen bonds at the sites of Thr24, Thr26, Cys145, Ser144, Leu141, His163, Gln189. Previous studies found His41, Gly143, Ser144, Cys145, His163, and Glu166 make contributions to interact with small molecular ligands through hydrogen bonds (Sabbah et al., 2021). Together, the dominant residue of 3CLpro does provide theoretical guidance for further design of molecules with greater binding capacity and stronger inhibitory abilities.

Previous studies suggest that galloyl substitution is critical for 3CLpro inhibition of tea catechins and theaflavins (Henss et al., 2021; Zhu and Xie, 2020). EC-C (1) is a bioactive catechin derivative with a...
caffeoyl group substituted at 3-OH other than a normal galloyl substitution at 3-OH such as those of ECG or ECGG. Our previous studies showed that this caffeoyl substitution enhanced its bioactivities (Wang et al., 2017; Zhang et al., 2018), which is consistent with the present result (Fig. 3, Fig. S3-5).

Previous studies also suggest that additional hydroxyl groups at the B ring might enhance catechins’ protein binding capacity (Liu et al., 2021). Although the present study did not show this trend with a stronger inhibition of ECG (IC$_{50}$ = 71.78 ± 8.36 µM) than that of ECGG (IC$_{50}$ > 100 µM) (Fig. S8), it is consistent with the α-amylase inhibition activity in which the catechol-type catechins were stronger than the pyrogallol-type catechins (Xiao et al., 2013).

Zijuanin C (2) and zijuanin D (3) (C-3′, C-3′′ isomers), (-)-8-(5′′-S)-N-ethyl-2-pyrrolidinone-3-O-cinnamoylpeicatechin (10) and (-)-8-(5′′-R)-N-ethyl-2-pyrrolidinone-3-O-cinnamoylpeicatechin (11) (C-5′′ isomers) are stereoisomers (Fig. 1), among which 2 and 10 can inhibit 3CLpro, while 3 and 11 cannot, suggesting that the stereoisomers of these catechins do affect their activities. Similarly, the activity of GCG (IC$_{50}$ = 3.38 ± 0.48 µM) is far stronger than its isomer EGCG, which is consistent with previous studies (Nguyen et al., 2012; Zha and Xie, 2020). Importantly, heating is an important processing procedure for tea production, which can lead to transforming EGCG to GCG (Zhou et al., 2018) and other important catechins (Zhang et al., 2021), thus enhancing various bioactivities of tea (Zhou et al., 2018) as well as the potential SARS-CoV-2 inhibition.

Although the major tea catechin EGCG showed weaker 3CLpro inhibition, it was reported to inhibit SARS-CoV-2 infections through different mechanisms (Henss et al., 2021; Zhang et al., 2021). Severe SARS-CoV-2 infection and high mortality are mainly caused by cytokine storm and inflammation, triggering a huge burden of oxidative imbalance on the immune system (El-Missiry et al., 2020; Zhang et al., 2021). As such, antioxidant, anti-inflammatory and immunity-enhancing therapies have become promising approaches to effectively treat COVID-19, contributed greatly by Traditional Chinese Medicine in China (Li et al., 2020). Various tea products (green, black, oolong, and roasted teas) contain lines of polyphenols. The major tea catechins EGCG, theasensin A, and gallated theaflavins exhibit viral prophylactic effects possibly through maintaining the redox homeostasis (Bao, G.H., Xu, J., Hu, F.L., Wan, X.C., Yu, M., Bao, G.H, 2013. EGCG inhibit 3CLpro activity intracellularly, among which EC-C and D (6), ECG, and GCG showed stronger inhibition. The extracellular 3CLpro IC$_{50}$ values of 1, zijuanin C (2), etc-pyrrolidinone C and D (6), etc-pyrrolidinone F (8), GCG, and ECG are 1.58 ± 0.21 µM, 41.2 ± 3.56 µM, 0.90 ± 0.03 µM, 46.71 ± 10.50 µM, 3.38 ± 0.48 µM, and 71.78 ± 8.36 µM, respectively. The K$_{D}$ values determined by SPR are 4.29 µM for 1, 3.46 µM for 6, and 3.36 µM for GCG. Our results indicate that the ester substitution of 3-OH and the configurations at position C-3, C-3′, C-3′′, and C-5′′ could affect the 3CLpro inhibition activity, suggesting that further structural modification could be conducted at 3-OH as well as the configuration changes at C-3, C-3′, C-3′′, and C-5′′.

Author Contributions

G.-H.B. and S.-Y.L. designed the experiments. S.-Y.L. and W.W. did most of the experiments. G.-H.B., J.-P.K., and P. Z. did some experiments. S.-Y.L. and G.-H.B. wrote the paper. G.-X.C. and G.-H.B. contribute to funding acquisition.

Declaration of Competing Interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

Guan-Hu Bao reports financial support was provided by National Natural Science Foundation of China. Guan-Hu Bao has patent #a new 3CL protease inhibitor preparation and use thereof CN202110687240.3 pending to China Patent.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153853.

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