Epigallocatechin Gallate Inhibits the Uridylate-Specific Endoribonuclease Nsp15 and Efficiently Neutralizes the SARS-CoV-2 Strain

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ABSTRACT: SARS-CoV-2, the coronavirus strain that initiated the COVID-19 pandemic, and its subsequent variants present challenges to vaccine development and treatment. As the coronavirus evades the host innate immune response at the initial stage of infection, the disease can have a long nonsymptomatic period. The uridylate-specific endoribonuclease Nsp15 processes the viral genome for replication and cleaves the polyU sequence in the viral RNA to interfere with the host immune system. This study screened natural compounds in vitro to identify inhibitors against Nsp15 from SARS-CoV-2. Three natural compounds, epigallocatechin gallate (EGCG), baicalin, and quercetin, were identified as potential inhibitors. Potent antiviral activity of EGCG was confirmed in plaque reduction neutralization tests with a SARS-CoV-2 strain (PRNT50 = 0.20 μM). Because the compound has been used as a functional food ingredient due to its beneficial health effects, we theorize that this natural compound may help inhibit viral replication while minimizing safety issues.

KEYWORDS: EGCG, green tea extract, COVID-19, SARS-CoV-2, coronavirus, Nsp15, endoribonuclease

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the worldwide outbreak of COVID-19. Limitations in available vaccines and established drugs against SARS-CoV-2 have made the treatment of this disease challenging. Because the virus evades the host innate immune response at the initial stage of infection, this disease can have a long nonsymptomatic stage. SARS-CoV-2 is a single-stranded, positive-sense RNA virus belonging to Family Coronaviridae and Order Nidovirales. The viral positive-sense RNA genome is primarily used in host cells as mRNA to translate large proteins to form 15–16 nonstructural proteins. The non-structural proteins mediate whole-genome and subgenome-length replication of mRNAs that encode structural and nonstructural proteins.

Among 16 nonstructural proteins, Nsp15 contains uridylate-specific endoribonuclease (EndoU) in the catalytic C-terminal domain. The viral EndoU was named nidovirus EndoU (NendoU) because it has only been found in Nidovirales. The crystal structure of Nsp15 from SARS-CoV-2 was determined at a high resolution. Nsp15 consists of three domains: the N-terminal oligomerization domain, the middle domain, and the C-terminal catalytic domain. The crystal structure studies further revealed a hexameric assembly comprising two trimers with an unprecedented spatial arrangement.

NendoU hydrolyzes the phosphodiester bonds at uridine sites of single- and double-stranded RNA, with 2′,3′-cyclic phosphodiester and 5′-hydroxyl termini produced. The host innate immune system employs the pattern recognition receptor MDA5 to recognize the polyU sequence in negative-sense viral RNA, which is replicated from polyA sequence in viral RNA. Nsp15 cleaves its own negative-sense RNA to limit the accumulation of the polyU-containing sequence in cells and thus evade the innate immune response. For example, Nsp15 reduces viral RNA in host cells and prevents RNA-activated antiviral responses by blocking the formation of cytoplasmic stress granules with antiviral functions. Nsp15 also has an essential role in the replication of coronaviruses in processing of the viral genome. Deletion of Nsp15 significantly decreases viral replication. Thus, the inhibition of Nsp15 activity may be a promising approach for preventing and controlling viral infection, by interrupting replication of the viral genome and activating the host innate immune response at the nonsymptomatic stage.

This study screened compounds inhibiting SARS-CoV-2 Nsp15 from a natural compound library. The screening initially identified three candidates as direct inhibitors of Nsp15. The top two compounds were subjected to an in vitro plaque assay with a SARS-CoV-2 strain isolated from human patients, and the neutralization activity of epigallocatechin gallate (EGCG) was confirmed. This study makes a case for the potential of EGCG in various dosing forms in the prevention and treatment of COVID-19.
Materials and Methods

Plasmid Construction and Protein Purification. The gene encoding Nsp15 in SARS-CoV-2 was synthesized by Bioneer Corp. (Daejeon, Korea) based on the sequence used in a previous study.7 The nsp15 gene was inserted into the pProEx-HT vector with a hexahistidine tag and tobacco etch virus cleavage site at the N terminus (Thermo Fisher Scientific, MA, USA). The plasmids were transformed into the Escherichia coli BL21 (DE3) strain. The transformed cells were cultured in 4 L of Terrific Broth medium containing 100 μg/mL ampicillin at 37 °C until an OD600 of 1.0 was measured, and then, the expression of protein was induced using 0.5 mM isopropyl β-d-1-thio-galactopyranoside at 16 °C for 20 h. After harvesting the cells by centrifugation, the cells were resuspended in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 5% (v/v) glycerol, and 2 mM 2-mercaptoethanol. Then, the cells were disrupted by sonication, and subsequently, the cell debris was removed via centrifugation at 19,000g and 4 °C for 30 min. The cell lysate was loaded onto Ni-NTA affinity agarose resin (GE Healthcare, IL, USA). The target protein was eluted with lysis buffer containing 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 5% (v/v) glycerol, and 2 mM 2-mercaptoethanol. The purified protein was concentrated and stored at −80 °C.

Endoribonuclease Assay. We employed a real-time endoribonuclease assay using an oligonucleotide substrate containing carboxyfluorescein (FAM) fluorophore at the 5′ terminus and tetramethylrhodamine (TAMRA) at the 3′ terminus.13 The oligonucleotide substrate 5′-FAM-dArUdAdA-TAMRA-3′ was synthesized by Bioneer Corp. (Daejeon, Korea). The substrate (2 μM) was used with 1 μM Nsp15 in a buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM MnCl₂, and 1 mM dithiothreitol. All chemicals used for screening are described in Table S1. The >95% EGCG and baicalein were purchased from Sigma-Aldrich (MO, USA). The initial screening using the natural compound library was conducted with 20 μM of each compound. The selected natural compounds were dissolved in dimethyl sulfoxide (baicalin, baicalein, and quercetin) or water (green tea extract and EGCG) and diluted to various concentrations for each reaction. The emission of the FAM fluorescence signal was detected with a 492 nm excitation wavelength and a 518 nm emission wavelength for 1 h using a SpectraMax i3x instrument (Molecular Devices, LLC., CA, USA). Reaction velocities were calculated with the slope of linear regression [relative fluorescence unit (RFU)/sec], and half maximal inhibitory concentration (IC₅₀) values with standard error (SE) were calculated with the four-parameter logistic curve (4PL) using Prism 8 software (GraphPad, CA, USA).

Plaque Reduction Neutralization Test with SARS-CoV-2. SARS-CoV-2 (NCCP 43326) provided by the Korea Disease Control and Prevention Agency (KDCA; formerly known as KCDC)14 was preincubated with serially diluted serum-free medium (Eagle’s minimum essential medium [EMEM]) containing green tea extract, EGCG, or baicalin for 30 min at 37 °C. Then, Vero cells were infected with preincubated SARS-CoV-2 for 30 min at room temperature and overlaid with 0.8% low-melting-point agarose. At 72 h after SARS-CoV-2 infection, the cells were fixed with 10% formalin and stained with 0.05% crystal violet to count the viral plaques. All procedures were performed in biosafety level-3 (BSL-3) facilities.

Molecular Docking Study. The molecular docking of Nsp15 with EGCG was elucidated using AutoDock Vina.15 The crystal structure of Nsp15 from SARS-CoV-2 (PDB: 6VWW) was used as the search model, and a grid box for the ligand docking site covered the active site pocket and adjacent areas. The best-suited conformation with the lowest root-mean-square deviation value was selected to calculate the binding energy between Nsp15 and EGCG using the same software. The interactions between Nsp15 and EGCG were analyzed using the Protein–Ligand Interaction Profiler (PLIP) web server.16
RESULTS

Screening for Inhibitors of Nsp15 Endoribonuclease in a Natural Compound Library. We synthesized the nsp15 gene from SARS-CoV-2 using a custom gene synthesis service and overproduced the protein in an E. coli expression system. We obtained the protein in soluble form when the cells were induced at 16 °C. The resulting protein exhibited endoribonuclease activity when we applied a previously established assay method using 5′-FAM-dArUdAdAdA-TAMRA-3′ as a substrate.13 When the synthetic substrate is cleaved at the rU site by Nsp15, the liberated FAM from TAMRA should emit a fluorescence signal at 512 nm. We used a compound library, consisting mainly of natural compounds, for screening (Figure 1A). The initial screening identified green tea extract, baicalin, and quercetin as candidate inhibitors (Figure 1B). The three candidate compounds completely or nearly abolished the activity of Nsp15 when each compound was applied at 20 μM. Notably, the preincubation of baicalin with the Nsp15 enzyme doubled the inhibitory effect (Figure 2), which was reflective of a slow binding pattern. The conformational flexibility of Nsp15 might have been associated with this preincubation effect of the inhibitor.

Inhibitory Effect of EGCG. The green tea extract strongly inhibited the Nsp15 enzyme from SARS-CoV-2 with an IC50 value of 2.54 μg/mL (Figure 3, Table 1). The green tea extract that we used in the screening contained EGCG, epigallocatechin, and epicatechin gallate. To analyze which compound in the green tea extract is mainly responsible for the inhibition of Nsp15, we first tested pure EGCG (>95% purity) in the same Nsp15 endoribonuclease assay because EGCG was the major ingredient in the green tea extract17,18 and dominates the water-soluble compounds in regular green tea. Pure EGCG inhibited the Nsp15 enzyme, as evidenced by the measured IC50 value, which was as low as 0.74 μg/mL or 1.62 μM (Figure 3, Table 1). The results showed that pure EGCG had an inhibitory activity that was approximately three times more potent than the activity of green tea extract when the same masses were applied. As the composition of EGCG in the green tea extract was approximately 50%,17,18 the inhibitory activity of EGCG could sufficiently account for the effect of the green tea extract. Thus, our results indicate that the inhibitory effect of the green tea extract resulted from EGCG.

Inhibitory Effects of Baicalin, Baicalein, and Quercetin. Baicalin is a glucuronide of baicalein that is found in Scutellaria baicalensis, Scutellaria lateriflora, and Scutellaria

![Figure 2](image-url). Effect of preincubating baicalin with the Nsp15 enzyme. Real-time fluorescence endoribonuclease assays were performed to observe the effect of preincubating baicalin with SARS-CoV-2 Nsp15. The y-axis represents the RFU, and the x-axis indicates reaction time.

![Figure 3](image-url). Inhibitory effects of green tea extract, EGCG, and baicalin on Nsp15. Real-time fluorescence endoribonuclease assays were performed in triplicate to observe the concentration-dependent inhibitory effects of selected natural compounds against SARS-CoV-2 Nsp15.

| compound          | IC50 ± SE*       |
|-------------------|------------------|
| green tea extract | 2.54 ± 0.45 μg/mL|
| EGCG              | 1.62 ± 0.36 μM (0.74 μg/mL) |
| Baicalin          | 7.98 ± 1.46 μM (3.56 μg/mL) |

Table 1. IC50 Values of Three Natural Compounds against Nsp15

*The IC50 values were calculated after 60 min of reaction based on real-time fluorescence endoribonuclease assays (see Figure 3). SE = standard error obtained from triplicate experiments.
Antiviral Activities of Green Tea Extract, EGCG, and Baicalin against SARS-CoV-2 in Cells. Although quercetin and baicalein exhibited inhibitory activities against the Nsp15 enzyme, we excluded quercetin and baicalein in the following work because of their lower inhibitory activity levels. The poor water solubilities of quercetin and baicalein further discouraged performing the following experiments with live coronavirus. To test the antiviral effects of the green tea extract, EGCG, and baicalin, we performed a plaque reduction neutralization test (PRNT) using SARS-CoV-2 isolated from COVID-19 patients. The PRNT is the standard method for quantifying circulating levels of the antiviral neutralizing antibody.23 Vero cells were infected with SARS-CoV-2 preincubated with varying concentrations of green tea extract, EGCG, or baicalin in EMEM. To measure the live viral titer, we counted the number of viral plaques. The green tea extract and EGCG had half neutralization effect concentrations (PRNT$_{50}$) of 0.24 μg/mL and 0.20 μM (0.092 μg/mL), respectively (Figure 4). As observed from the results of the Nsp15 endoribonuclease assay, considering the content of EGCG in the green tea extract used in this study, the EGCG in the extract is the major contributor to the antiviral activity exhibited by the extract. Baicalin exhibited a limited antiviral effect in comparison with EGCG (PRNT$_{50}$ = 83.3 μM [37.2 μg/mL]) (Figure 4). The poor solubility of baicalin in the experimental medium might explain the lower antiviral effect expected from the IC$_{50}$ value in the endoribonuclease assay with Nsp15.

Nsp15 and EGCG Docking Model. To gain molecular insights into Nsp15 binding, we generated a docking model using the crystal structure of Nsp15 from SARS-CoV-2 (PDB: 6VWW).15 The docking model generated with AutoDock Vina software15 ranked EGCG binding at the active site of Nsp15 at the top. The binding energy of this molecular docking was −6.9 kcal/mol. The EGCG molecule appeared to be tightly bound to the active site of Nsp15 (Figure 5A). According to the docking model, the many hydroxyl groups of EGCG interact with all key residues at the active site of Nsp15. We analyzed the EGCG–Nsp15 interactions in the docking model using the PLIP web server.16 The bound EGCG molecule has hydrophobic interactions with Lys290, Val292, Tyr343, and Leu346 at the Nsp15 active site and forms hydrogen bonds with His235, Gly248, His294, Ser294, and Thr341 at the site. The carbonyl group of EGCG has a polar interaction with His235, Gly248, His250, Lys290, Tyr343, and Leu346 at the Nsp15 active site (Figure 5A).

Figure 4. In vitro antiviral activities of green tea extract, EGCG, and baicalin against the SARS-CoV-2 strain. The anti-SARS-CoV-2 activities of the compounds were tested using a PRNT. The y-axis represents the neutralization percentage, and the x-axis indicates the concentration of each compound. Concentrations of green tea extract, EGCG, and baicalin resulting in a neutralization percentage greater than 50% were considered to be the PRNT$_{50}$ values. The mean and standard deviation values were calculated from three replicate experiments. All experiments were performed in biosafety level-3 facilities.

Baicalin exhibited a limited antiviral effect (Figure 5A). According to the docking model, the many hydroxyl groups of EGCG interact with all key residues at the active site of Nsp15. We analyzed the EGCG–Nsp15 interactions in the docking model using the PLIP web server.16 The bound EGCG molecule has hydrophobic interactions with Lys290, Val292, Tyr343, and Leu346 at the Nsp15 active site and forms hydrogen bonds with His235, Gly248, His250, Lys290, Ser294, and Thr341 at the site. The carbonyl group of EGCG has a polar interaction with Lys290 (Figure 5B). In Nsp15 enzymes from other coronaviruses, residues corresponding to His235, His250, and Lys290 make up a His–His–Lys triad that plays a critical role in endoribonuclease activity. The other residues at the active site are also involved in oligomer formation and enzymatic activity.5,24,25 The gallate moiety (D-ring) and B-ring are stacked by a π interaction, and the two stacked rings fit well into the active site pocket (Figure 5). The docking model indicates the importance of the gallate moiety in EGCG for the inhibition of Nsp15.
Recently, Ohgitani et al. observed that brewed green tea fully inhibited Nsp15 enzymatic activity when 20 mg of dried green tea leaves were steeped in 100 mL of water at 70 °C (Figure S2). These findings indicate that EGCG and EGCG-containing drinks are excellent materials for controlling the replication of the coronavirus.

EGCG exhibited antiviral activity in the plaque assay using the live SARS-CoV-2 strain that was more potent than its Nsp15 inhibitory activity. We believe that the higher antiviral activity of EGCG may result from the binding of EGCG to other viral proteins or inhibitory effects on these proteins. Recent studies reported that EGCG exhibited inhibitory activity against 3CLpro from SARS-CoV-2. Structural docking studies further suggested that the spike protein, PLpro, and RNA-dependent RNA polymerase may be possible targets of EGCG. These findings indicate that the antiviral effect of EGCG constitutes multiple inhibitions of viral enzymes acting synergistically.

We need to be concerned about treatment resistance in coronavirus variants. However, multiple-target drugs or a combination of structurally different drugs can be used to overcome the resistance because effective resistance requires simultaneous mutations in the multiple targeted proteins or multiple mutations in a single-target protein. Importantly, EGCG exhibited a strong antiviral effect at the sub-micromolar range, and baicalin increased the antiviral effect of EGCG at the sub-micromolar range when SARS-CoV-2 was treated with both EGCG and baicalin (Figure S3). Thus, co-treatment with EGCG and baicalin may be promising for maximizing the antiviral effect and minimizing resistance to the treatment.

Previous research has suggested that EGCG has health benefits, including antiaging, anticancer, and anticardiovascular effects. EGCG is sold commercially as a dietary supplement and has high water solubility. However, EGCG resulted in low pharmacokinetic parameters in a rat model when taken orally, and excess uptake of EGCG may cause liver injury or adverse health effects. SARS-CoV-2 interacts with angiotensin-converting enzyme 2 in the small intestinal epithelia and induces an imbalance in the composition and diversity of intestinal microbiota, resulting in inflammation and diarrhea. As EGCG shows strong antiviral activity even at low concentrations, we may expect that the coronavirus in the digestive tract would be cleared by direct contact with EGCG if green tea containing EGCG was consumed continuously at levels lower than the tolerable upper intake level.

Alternative dosing methods can be employed to induce the normal innate immune response for prevention or curative treatment at the early stage of coronaviral infection. In previous research, EGCG was found to be well absorbed by the mucosa, leading to the prevention of infection by the influenza virus, and EGCG blocked the propagation of other coronaviruses. We suggest that EGCG can be more effective in the prevention of infection by SARS-CoV-2 or other coronavirus-related diseases if it is used in a mouth rinse, nasal wash solution, or nasal spray. A fabric containing a combination of natural compounds would help kill or inactivate the virus.

In conclusion, EGCG is a good natural compound for inhibiting Nsp15 from SARS-CoV-2, and it exhibits efficient antiviral activity against SARS-CoV-2 at the sub-micromolar range, presumably due to multiple inhibitions of viral proteins. With further investigation, EGCG could be applied to reduce the time and processes required for developing therapeutic drugs and preventive treatments in this urgent situation.

DISCUSSION

The implementation of effective and safe natural compounds can help reduce the time and processes required to develop therapeutic drugs and preventive treatments. Herein, we screened and investigated natural compounds as direct inhibitors of the Nsp15 EndoU from SARS-CoV-2. All of the inhibitors found in this study are polyphenolic compounds typically included in the human diet in amounts varying from approximately 1 to 2 g. A negative correlation between a moderate dose of EGCG completely neutralized the replication of SARS-CoV-2 in cells. The effective concentration of EGCG was very low compared to the EGCG concentration in regular brewed green tea (70 mg of EGCG in 100 g of brewed green tea; 0.7 mg/mL EGCG concentration). Recent studies reported that EGCG exhibited inhibitory activity against 3CLpro from SARS-CoV-2. Structural docking studies further suggested that the spike protein, PLpro, and RNA-dependent RNA polymerase may be possible targets of EGCG. These findings indicate that the antiviral effect of EGCG constitutes multiple inhibitions of viral enzymes acting synergistically.

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Inhibitory effects of baicalein, quercetin, and brewed green tea on Nsp15; In vitro antiviral activity of the green tea extract with or without baicalein against the SARS-CoV-2 strain; and complete list of the compounds in the natural compound library (PDF)

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Notes
The authors declare no competing financial interest.

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