Epigallocatechin-3-Gallate (EGCG) Inhibits SARS-CoV-2 Infection in Primate Epithelial Cells

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ABSTRACT

SARS-CoV-2, the novel coronavirus responsible for the COVID-19 pandemic, caused >26 million cases in the United States and >437,000 deaths as of Jan 30, 2021. Worldwide by that date, there had been 102 million cases of infections, and deaths had climbed to 2.21 million. Mutated variants of SARS-CoV-2 that have emerged from the United Kingdom, Brazil, and South Africa are associated with higher transmission rates and associated deaths. Therefore, novel therapeutic and prophylactic methods against SARS-CoV-2 are in urgent need. While some antiviral drugs, such as Remdesivir, provide relief to certain patient populations, other existing antiviral drugs or combinations of FDA approved pharmaceuticals have yet to show clinical efficacy against COVID-19. Compounds that possess strong and broad antiviral properties with different mechanisms of action against respiratory viruses may provide novel approaches to combat SARS-CoV-2 and its variants, especially if the compounds are classified as generally recognized as safe (GRAS). A large body of evidence indicates a promising potential for the use of epigallocatechin-3-gallate (EGCG) and its derivatives as effective agents against infections from a wide range of pathogenic viruses. However, EGCG or its derivatives have not been tested directly against SARS-CoV-2. The current study was designed to evaluate the potential antiviral activity of EGCG against SARS-CoV-2 infection in primate epithelial cells. Methods applied in the study include cytopathic effect (CPE) assay and virus yield reduction (VYR) assays using Vero 76 (green monkey epithelial cells) and Caco-2 (human epithelial cells) cell lines, respectively. The results demonstrated that EGCG at 0.27 µg/ml (0.59 µM) inhibited SARS-CoV-2 infection in Vero 76 cells by 50% (i.e., EC50=0.27 µg/ml). EGCG also inhibited SARS-CoV-2 infection in Caco-2 cells with EC90=28 µg/ml (61 µM). These results, to the best of our knowledge, are the first observations on the antiviral activities of EGCG against SARS-CoV-2, and suggest that EGCG and its derivatives could be used to combat COVID-19 and other respiratory viral infection-induced illness, pending in vivo and clinical studies.

Introduction

Previous studies indicated that EGCG, a major hydrophilic component of extracts from leaves of the *Camellia sinensis* (tea) plant, and its hydrophobic derivatives (especially EGCG-palmitate, or EC16), could be potential candidates for antiviral and virucidal material (1-19, reviewed in 20). Importantly, EGCG and lipophilic EGCG (such as EC16) are classified as non-toxic food additives, considered by the US FDA as a generally recognized as safe (GRAS) food additive (FDA GRAS Notice 772).

Accumulating data indicate that EGCG and its hydrophobic derivatives may fill gaps in the area of prevention and treatment of influenza virus infection, by multi-target mechanisms [21]. EGCG inactivates virus particles by direct binding to viral coat proteins, and it inhibits viral-receptor binding, endosome formation and internalization, nuclear uptake, and transcription of the viral genome [22].

EGCG at micromolar (μM) levels inhibits the infectivity of a diverse group of enveloped and non-enveloped viruses by interrupting viral attachment to cell membrane receptors [8]. We reported that inactivation of HSV-1 was through non-specific binding of EGCG to viral surface proteins [18,19]. EGCG at μM levels dose-dependently inhibited Ebola virus infectivity in human cells [9]. Either green tea extract or pure EGCG inhibited the infectivity of hepatitis B virus (HBV) in HepG2-N10 cell culture [20]. At concentrations as low as 0.002 μM, EGCG inhibited hepatitis C virus (HCV) entry into hepatoma cell lines and primary human hepatocytes, by interruption of viral attachment to host cells [20]. Importantly, EGCG at physiologically achievable levels of 1 to 10 μg/mL (2.17-21.7 μM) effectively inhibited HCV cell-to-cell spread, making EGCG a potential solution to prevent and treat Hepatitis C [22]. EGCG's is direct binding to the virion leads to a bulging of the viral envelope. This structural change of the virion might contribute to the loss of the virion's ability to attach to cell surface receptors [21]. This mechanism could also be responsible for preventing virion entry of HBV [23]. EGCG shows high-affinity binding to CD4 protein, thereby inhibiting the binding of HIV vgp120 to human CD4$^+$ cells [24-26]. EGCG binds to influenza A and B viral hemagglutinin (HA) and sialic acid on the cell membrane, and alters the membrane properties, leading to inhibition of viral entry [8,28-30]. EGCG also binds to neuraminidase (NA), at a different binding site (residue 430) than other drugs, and reduces the activity of releasing new viral particles from infected cells [31,32]. EGCG reduces viral replication by interfering with the association between viral dsRNA and NS1 by binding to NS1 protein [33]. Importantly, in human nasal epithelial cells, the antioxidant activity of EGCG combined with upregulation of Nrf-2 expression contributes to inhibition of viral infection and replication, through EGCG-induced Nrf-2 expression significantly inhibiting viral entry [34]. EGCG also has an inhibitory effect on influenza A RNA polymerase activity [35]. Specific binding between EGCG and viral RNA polymerase has been confirmed, and EGCG was shown to chelate critical manganese ions in the active site of the enzyme [36]. These properties could explain our data showing EGCG prevents H1N1 infection in cell culture [37]. In addition, the antiviral properties of EGCG have a wide species spectrum, and EGCG has been shown to be effective against most human viruses tested [8].

As a potent antioxidant, water-soluble EGCG is quickly self-oxidized in an oxygen-rich environment. Thus, it is difficult to maintain activity in a liquid formulation. Therefore, EGCG derivatives were studied to search for a candidate form of EGCG that was physically stable, and more active against viruses, which could be suitable for a new generation of EGCG-derived small molecules active against viral infections [38]. A group of patented lipophilic EGCG compounds (US composition patent 8076484) has been tested in multiple *in vitro* and *in vivo* models. Kaihatsu's group identified a lipid-soluble EGCG derivative, EGCG-palmitate (EC16), as the most active form of EGCG for influenza virus inactivation, being 44-fold more effective than hydrophilic EGCG [4]. Similarly, previous studies from our group demonstrated that

EC16 is more potent than EGCG for herpes simplex virus 1 (HSV-1) inactivation, and for herpes labialis, the clinical outcome from topical application of the EC16-containing formulation AverTeaX was more effective than other topical medications [11,12,19]. We also found that the virucidal activity of EC16 lasted for several hours after a single application on a clean surface [39]. Importantly, newly reported data from our group indicates that EC16 in a proprietary liquid formulation effectively protected MDCK epithelial cells from influenza virus infection [37]. These green tea polyphenols are able to inhibit SARS-CoV TMPRSS2 [40,41], N protein [42,43], 3C-like protease [44,45], and these observations suggest a multi-targeted, and thus potentially mutation-proof, mechanism of action against viruses, potentially including coronavirus.

In summary, the broad-spectrum of antiviral and virucidal properties of EGCG and EGCG-palmitate with multi-target mechanisms have been documented with encouraging efficacy results. The current study determined the potential antiviral activities of EGCG against SARS-CoV-2 infection in different primate cell lines.

Material and Methods

EGCG was provided from the Research Laboratory of Camellix, LLC (Augusta University, Augusta, GA). All other materials and methods were provided through a non-clinical evaluation agreement (NCEA) between the National Institute of Allergy & Infectious Diseases (NIAID) and Augusta University/Camellix, LLC. The experiments were performed in the Institute for Antiviral Research, Utah State University. The cytotoxicity tests were performed using a standard test method that exposed the cell culture to EGCG for the entire test period of antiviral assays. This method aims to induce the maximum cytotoxic effect possible by EGCG. In contrast, the antiviral tests described below exposed the cells to EGCG for 1 hr.

Primary CPE assay – Vero 76 cells

Confluent or near-confluent cell culture monolayers of Vero 76 cells were prepared in 96-well disposable microplates the day before testing. Cells were maintained in Minimum Essential Media (MEM) supplemented with 5% fetal bovine serum (FBS). For antiviral assays the same medium was used but FBS was reduced to 2% and supplemented with 50-μg/ml gentamicin. EGCG was dissolved in serum-free MEM at four serial $\log_{10}$ concentrations, usually 0.1, 1.0, 10, and 100 μg/ml. Five micro wells were used per dilution: three for infected cultures and two for uninfected EGCG toxicity assessment. Controls for the experiment consist of six microwells that were infected and not treated (virus controls) and six that were untreated and uninfected (cell controls) on every plate.

The dilutions of EGCG were incubated with SARS-CoV-2 (1:1) at ~60 CCID$_{50}$ (50% cell culture infectious dose) in 0.2 ml volume for 1 h prior to adding to the cell culture for 1 h absorption after removal of the growth media. The EGCG/SARS-CoV-2 mix was

then removed and 0.2 ml fresh MEM with 2% FBS and 50-µg/ml gentamicin was added to the cell culture. Medium devoid of virus was placed in toxicity control wells and cell control wells. Plates were incubated at 37°C with 5% CO_2 until marked (> 80%) CPE was observed in virus control wells. The plates were then stained with 0.011% neutral red for approximately two hours at 37°C in a 5% CO_2 incubator. The neutral red medium was removed by complete aspiration, and the cells rinsed 1X with phosphate buffered solution (PBS) to remove residual dye. The PBS was completely removed, and the retained neutral red was eluted with 50% Sorensen's citrate buffer/50% ethanol for at least 30 minutes. Neutral red dye penetrates into living cells, thus, the more intense the red color, the larger the number of viable cells present in the wells. The dye content in each well was quantified using a spectrophotometer at 540 nm wavelength, and dye content in each set of wells was converted to a percentage of dye present in untreated control wells using a Microsoft Excel computer-based spreadsheet and normalized based on the virus control. The 50% effective (EC_{50}, virus-inhibitory) concentrations and 50% cytotoxic (CC_{50}, cell-inhibitory) concentrations were then calculated by regression analysis. The quotient of CC_{50} divided by EC_{50} gives the selectivity index (SI) value.

Secondary VYR assay - virus yield reduction – Caco-2 cells
The VYR assay is a direct determination of the ability of EGCG to inhibit virus entry and replication. Caco-2 cells were maintained in MEM supplemented with 10% FBS. EGCG was dissolved in serum-free MEM in a series of 2 x dilutions from 100 µg/ml down to 0.78 µg/ml, and dilutions were incubated with SARS-CoV-2 (1:1) at 200 $CCID_{50}$ (50% cell culture infectious dose) in 0.1 ml volume for 1 h prior to adding to the cell culture for 1 h absorption. The EGCG/SARS-CoV-2 mix was removed and fresh MEM was added to the cell culture with 2% FBS, 50-µg/ml gentamicin for viral yield detection in comparison to untreated infection control. Five microwells were used per dilution: three for infected cultures and two for uninfected toxicity tests. Controls for the experiment consisted of six microwells that were infected but not treated (virus controls) and six that were untreated and uninfected (cell controls) on every plate. After 72 h, supernatants from each set of three replicate infected wells were pooled and a sample was tested immediately in a viral yield reduction assay. Virus yield in the presence of EGCG was titrated and compared to virus titers from the untreated virus controls. Titration of the viral samples was performed by endpoint dilution: serial 1/10 dilutions of virus were made and plated into 4 replicate wells containing fresh cell monolayers of Vero 76 cells. Plates were then incubated, and cells were scored for the presence or absence of virus after distinct CPE was observed, and the $CCID_{50}$ calculated using the Reed-Muench method [47]. The 90% (one log_{10}) effective concentration (EC_{90}) was calculated by regression analysis by plotting the log_{10} of the inhibitor concentration versus log_{10} of virus produced at each concentration. The quotient of CC_{50} divided by EC_{90} gives the selectivity index (SI) value.

Results
Primary CPE assay – Vero 76 cells
The cytopathic effect (CPE) assay results showed that EGCG inhibited 50% of SARS-CoV-2 infection in Vero cells at 0.27 µg/ml (i.e., EC50=0.000027% or 0.59 µM) (Table 1) after 1 hr exposure to EGCG, while 50% cytotoxicity (CC50) after the completion of antiviral assays (7-10 days) was apparent when the concentration of EGCG increased 8.5 times to 5.03 µM.

Secondary VYR assay - virus yield reduction – Caco-2 cells
The virus yield reduction (VYR)) assay results showed that EGCG inhibited 90% of SARS-CoV-2 infection in Caco-2 cells at 28 µg/ml (i.e., EC90=0.0028% or 61 µM) (Table 1), while 50% cytotoxicity (CC50) was not observed even when the EGCG concentration was increased to >100 µg/ml or 217 µM. Thus, the SI value could only be presented as >3.6 due to the low cytotoxicity of EGCG to Caco-2 cells.

Table 1: Inhibitory effects of EGCG on SARS-CoV-2 in two primate epithelial cells.

Vero 76 Cells	EC50	CC50	SI
	0.59 µM	5.03 µM	8.5
Caco-2 Cells	EC90	CC50	SI
	61 µM	>217 µM	>3.6

As shown in Table 2, EGCG at 50 µg/ml (0.005%), EGCG caused a further 1.8 log_{10} reduction in viral titer in comparison to 12.5 µg EGCG, equivalent to a 98.41% reduction of viral yield in Caco-2 cells. At 100 µg/ml (0.01%), EGCG resulted in >3.6 log_{10} reduction in virus titer in comparison to 12.5 µg EGCG, a >99.975% reduction of viral yield.

Table 2: Viral titers of Caco-2 cell culture after SARS-CoV-2 infection in the present of EGCG for 1 h before EGCG/virus was removed. Raw data generated from virus yield reduction assay expressing dose-respons of EGCG-induced viral infection in TCID50 values.

EGCG concentration (µg/ml)	Viral titer of EGCG-treated Cell Culture TCID50 in log_{10} values
100	<1.7
50	3.5
25	5.0
12.5	5.3

Discussion
The cytopathic effect (CPE) assay results showed that after a 1 hr exposure, EGCG was effective and blocked 50% of SARS-CoV-2 infection in Vero 76 cells at a very low concentration of 0.000027% (0.59 µM). Prolonged exposure to EGCG induced cytotoxicity in Vero 76 cells at 0.00023% or 5.03 µM, a near 10-fold higher concentration. However, this concentration of EGCG-associated cytotoxicity in Vero 76 cells was somewhat lower than previously published data. For example, our group reported that after 48 h incubation with 50 µM EGCG, cell proliferation rate was reduced to 79.68% in Vero cells (parental cell line of Vero 76 cells) [19]. This inconsistency could be due to the different assay methods, culture conditions and/or cell lines used.

In contrast, the observed CC50 for EGCG in Caco-2 cells, a cell line derived from human intestinal epithelial cells (colorectal adenocarcinoma), was >217 µM of EGCG, consistent with previously published data in human cells showing low cytotoxicity.

In general, EGCG and its derivatives are considered safe for oral and topical applications for human use. A group of patented lipophilic EGCG derivatives (US 8076484) has been tested in multiple *in vitro* and *in vivo* models without apparent toxicity [11,12,19,48,49]. In 2019, the US FDA approved the use of tea catechins palmitate (containing approximately 50% EGCG-palmitate) as a GRAS (generally recognized as safe) dietary ingredient (FDA GRAS Notice 772, Ref 50). It was found that tea catechin palmitate undergoes hydrolysis after consumption, consistent with our findings that EGCG-fatty esters are hydrolyzed to free EGCG after entering cells [5]. These studies concluded that the intended use of tea catechin palmitate meets the GRAS requirement [50]. Indeed, our previous data demonstrated that a 1 h incubation of Vero cells with 50 µM EGCG-palmitate only reduced cell viability by 0.043%, and reduced the cell growth by 10.91% after 48 h incubation [19]. Of note, the antiviral activity against herpes simplex virus 1 (HSV-1) of EGCG-palmitate is significantly higher than that of EGCG – 100% vs. 92.15% [19]. This difference in antiviral activity between EGCG-palmitate and EGCG was also reported by others [3].

Currently, methods that could be effective against novel pathogenic respiratory viruses such as SARS-CoV-2 or other types of or mutated coronavirus are not available. There is an urgent need to develop a fast acting, efficacious, economical strategy with a completely different mechanism of action to protect airway epithelial cells from viral entry; with multiple targets for effect against viral entry and replication and thereby potentially mutation-proof; agents to rapidly inactivate a broad-spectrum of respiratory viruses; and agents with the above properties but without toxic side effect.

Since the major point of host entry for SARS-CoV-2 is *via* nasal epithelial cells, intranasal-applied therapeutics could be highly effective [51]. Based on *in vitro* and *in vivo* evidence, we hypothesize that EGCG and its hydrophobic derivatives possess strong antiviral activity against human coronaviruses, with potential to become therapeutic and prophylactic agents against infections of SARS-CoV-2 and its mutants. While the mechanisms of SARS-CoV-2 transmission are still under investigation, aerosol transmission is a known significant route for viral entry to infect humans [51]. Our previous studies demonstrated that EGCG in a formulation suitable for nasal application is effective against influenza virus H1N1 [37].

Here we report, for the first time according to the best of our knowledge, that EGCG is able to inhibit SARS-CoV-2 infection in primate epithelial cells, include human cells, at doses not associate with toxicity. These results demonstrate that this class of compounds derived from green tea leaf extracts are potential agents for new drug development against a wide spectrum of respiratory viruses, including novel coronavirus and its mutated variants, pending further investigations.

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