Effect of Novel Formulations using Lipophilic Epigallocatechin-3-Gallate against Influenza Virus Infection

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ABSTRACT

Influenza virus infection is associated with high morbidity and mortality, and effective prophylactic and therapeutic methods to reduce infection outbreaks are lacking. Vaccination and current prevention/treatment approaches are not associated with a decline in morbidity or mortality, either globally or in the United States. Neither vaccination nor current anti-viral drugs prevent viral entry into the body and host cells, but rather target post-infection events in individuals. Hand washing and sanitizing do not prevent aerosol viral transmission, which accounts for the majority of influenza virus infections. Therefore, protection of the epithelia of the respiratory tract from viral entry is a significant gap in preventive strategies that needs to be filled. We hypothesize that lipophilic epigallocatechin-3-gallate (L-EGCG), and EGCG-palmitate (EC16) in particular, is able to fill this gap and become a first-in-class prophylactic and therapeutic approach against influenza virus infection. The objective of the current study was to investigate a proof-of-concept for the use of EC16 to prevent and treat influenza virus infection. The experimental design included direct contact of formulations containing EC16 with H1N1 influenza virus prior to infection assay (TCID50) in MDCK cells, incubation of cells with EC16 formulations either before or after H1N1 viral infection (without direct formulation contact with the virus), and coating the cell surface with EC16 formulations prior to H1N1 viral infection, followed by TCID50 assays. The results demonstrated that at a 0.1% concentration, EC16 formulations were effective (>95%) in blocking H1N1 infection regardless of direct contact with the virus. In conclusion, formulations containing EC16 could be an effective prophylactic and therapeutic approach to combat influenza infection in the respiratory tract, pending further in vitro and in vivo studies.

Keywords

Influenza virus, EGCG, Seasonal flu prevention, Lipophilic EGCG, H1N1.

Introduction

According to the World Health Organization (WHO), seasonal flu caused by human influenza viruses A and B is responsible each year for approximately 500,000 deaths worldwide. In the United States, recent annual influenza-associated deaths ranged from 12,000 (2011-2012 season) to 56,000 (2012 -2013 season), and despite enforced vaccination and education in flu prevention, flu-associated pediatric deaths in the United States during the 2017-2018 season reached a record high of 180. Total deaths also set a recent record of more than 80,000, as did the number of hospitalizations (710,000) in the United States (US CDC).

Flu prevention and treatment have several complicated issues that need to be addressed more effectively. On the prevention front, there is a lack of effective methods to prevent viral entry into the upper respiratory epithelial cells, which is the route responsible for most influenza viral infections in humans. Hand hygiene education and practice are not associated with a decreased morbidity or mortality linked to seasonal flu, possibly due to the short-lasting effects of washing or alcohol prior to recontamination. At post-viral entry stages, vaccines and anti-viral drugs are the primary methods to control symptoms. Despite vaccination having been referred to as a "prevention" method, an individual must be infected by the virus in order for vaccination to have an effect. Major problems with vaccines include frequent miss-matches to the most pathogenic influenza viral strain(s) in a season, and populations unable to mount an effective immune response,

leading to influenza outbreaks at epidemic or pandemic levels.

In the area of treatment, antiviral drugs are designed to target a single viral protein component in order to interfere with the viral infection cycle after viral entry into the epithelial cells of the respiratory tract. There are two classes of anti-influenza drugs that have been approved specifically for treating influenza symptoms - adamantanes (M2 ion channel inhibitors) and neuraminidase inhibitors. However, adamantanes are no longer recommended due to a significant worldwide increase in resistant viral strains [1]. The clinical efficacy of these antiviral drugs has been questioned by researchers and clinicians due to the recently unveiled disappointing clinical outcomes for oseltamivir (Tamiflu), a neuraminidase inhibitor that blocks new virion release. Oseltamivir only resulted in a 20-hour mean reduction in symptoms without a decrease in the likelihood of developing pneumonia, hospital admissions, and complications requiring antibiotic treatment [2]. This former WHO-recommended first line essential medicine (core drug) against influenza was downgraded by WHO in 2017 to a complementary drug due to new evidence of low clinical efficacy.

Another major problem is that frequent mutations in the influenza virus genome reduce the effectiveness of antiviral drugs that target a single protein/enzyme of the virus as the mechanism of action by selection of resistant viral strains [3]. Drug-resistant virus variants have emerged for neuraminidase inhibitor antiviral drugs [4], including oseltamivir. Indeed, the majority of circulating influenza A viruses, especially H1N1 and H3N2, have developed resistance to these antiviral drugs (such as the H275Y mutation in the neuraminidase protein coding sequence of the 2009 pandemic H1N1), and an increasing number of drug resistant viral strains are being isolated due to the constantly changing genetic makeup of the virus [1].

Recently, the new anti-influenza drug, Xofluxa (baloxavir marboxil), has been developed and received US FDA approval; it is hoped that it may provide an additional treatment method against flu outbreaks. However, despite Xofluza targeting a different viral protein, cap-dependent endonuclease, the efficacy of this agent in reduction of symptoms is similar to oseltamivir [5]. This new addition of a single target antiviral drug not only has similar efficacy compare to other anti-influenza drugs, but also with time will likely induce new virus variants that can evade the drug mechanism.

In summary, due to the limited effects of currently available methods, the requirement for better strategies to combat a flu pandemic is several fold. There is a need to develop: a strategy to protect upper respiratory tract epithelial cells from viral entry; a multi-target agent against viral entry and replication; an agent to rapidly inactivate a broad-spectrum of influenza viruses (especially A and B) on contact; and an agent with the above properties but without the risk of developing resistant virus variants or toxic side effect.

These issues are not limited to influenza virus. In a 2018 WHO annual review of diseases prioritized under the Research and Development Blueprint, due to the potential public emergency and the absence of efficacious drugs/or vaccines, the WHO determined that there is an urgent need for accelerated research and development for better approaches of controlling Crimean-Congo hemorrhagic fever, Ebola viral disease and Marburg viral disease, Lassa Fever, MERS and SARS, Nipah and henipaviral diseases, Rift Valley fever, Zika disease, and disease X. These diseases are all caused by viruses (except Disease X, which will likely be caused by a highly pathogenic virus variant, i.e. a super virus). Thus, a broad-spectrum antiviral agent with the above properties would be highly desirable.

Accumulating data indicate that epigallocatechin-3-gallate (EGCG) isolated from green tea leaf extract, and especially lipophilic EGCG (L-EGCG) derivatives, may fill these gaps in the area of prevention and treatment of influenza virus infection [6]. A large body of research data indicates that the effects of EGCG against viral infection target multiple stages of the viral infection cycle [7]. In 2005, Song et al. reported that for both influenza A and B viruses, EGCG is able to inhibit hemagglutinin and neuraminidase activities, altering viral membrane properties, and inhibits viral RNA synthesis [8]. A mass spectrometry study showed that EGCG binds to neuraminidase at residue 430, which serves as a sialic acid binding site [9]. Thus, the viral internalization process can be interrupted by EGCG through physical damage to the viral envelope [10]. EGCG has also demonstrated an inhibitory effect on influenza A RNA polymerase activity [11]. Specific binding between EGCG and viral RNA polymerase has been confirmed, and EGCG chelates critical manganese ions in the active site of the enzyme [12]. As a potent antioxidant, the inhibitory effect of EGCG on influenza A virus is associated with suppression of viral infection-induced reactive oxygen species [13]. This evidence suggests that EGCG not only blocks viral entry into cells, but also interferes with viral replication intracellularly. In addition, the antiviral properties of EGCG have a wide spectrum effective against most viruses studied previously [14].

However, as a potent antioxidant, water-soluble EGCG is quickly self-oxidized in an oxygen-rich environment, making it difficult to maintain activity in a liquid formulation when applied either topically or systematically. Therefore, candidate derivatives of EGCG were studied in a search for a physically stable, lipid-soluble, and more active form of EGCG that could be suitable for a new generation of EGCG-based small molecules active against viral infections [15]. From various derivatives of EGCG, Kaihatsu's group identified a lipid-soluble EGCG, EGCG-mono-palmitate, as the most effective form of EGCG to inactivate influenza virus, being 44-fold more effective than water-soluble EGCG [16]. Previous studies from our group demonstrated that EGCG-palmitate (EC16) is more potent than EGCG for herpes simplex virus 1 (HSV-1) inactivation, and the clinical outcome from topical application of the EC16-containing formulation AverTeaX for herpes labialis was superior to other topical medications against herpes labialis [17-19].

In the current study, we conducted a proof-of-concept series of experiments to test the specific hypothesis that formulations containing EC16, either with or without direct contact with the virus, effectively block infection by H1N1 virus (a subtype of influenza a virus). The long-term goal of our effort is to develop topical formulations to protect the airway epithelia from influenza virus infection.

Materials and Methods

EC16 was purchased from Camellix, LLC (Augusta, GA). MDCK cells were obtained from American Type Culture Company (ATCC CCL-34, Manassas, VA) and stored in liquid nitrogen prior to use. H1N1 virus (A/Virginia, ATCC VR-1736, Manassas, VA) was also purchased from ATCC and stored at -80°C.

Infection of MDCK cells and TCID50 viral titer assay

MDCK cells were cultured in Minimum Essential Media (MEM, Life Technologies Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Heat inactivated, Neuromics, Edina, MN) and 1X penicillin, streptomycin, and amphotericin B (Corning, Corning, NY). The viral infection assay was performed in 96 well cell culture plates (tissue culture treated, Southern Labware, Cumming, GA) using MDCK cells that had reached confluency. A series dilution of H1N1 virus stock to 10^{-7} fold was prepared using MEM serum-free medium with antibiotics, and 100 µl of each viral mix dilution was loaded into wells with four replicate wells per dilution. After a one hour incubation, the viral dilutions were removed and 200 µl MEM serum-free medium with 0.2 µg/ml trypsin (Life Technologies Corporation, Carlsbad, CA) was added to the wells, followed by incubation at 35°C with 5% CO_2 for 4 days to allow a CPE (Cytopathic effect) to become visible. According to the TCID50 protocol and software [20], the number of wells showing CPE was entered into the calculation formula to determine the infection activity of the virus (titer). The viral titer without any EC16 treatment was set as 100%. The remaining viral infection titer from various EC16 treatments was determined and the percentage of the untreated infection rate was calculated.

Viral inactivation test

Formulations containing EC16 were made by dissolving EC16 in a proprietary polyol carrier (referred to as "carrier" hereafter) and then diluting with a mixture of MEM serum free medium and carrier to 0.01% (w/v) or 0.1% EC16 in 10% or 20% carrier. In a 2 ml micro centrifuge tube, 50 µl of H1N1 virus stock was added to 450 µl of a formulation containing EC16 and carrier. The tube was then closed and the contents mixed by shaking for 60 sec of direct contact, and the viral/EC16 mix was then immediately diluted 10X in MEM serum-free medium (100 µl mix to 900 µl MEM) in order to inactivate EC16, followed by series 10-fold dilutions to 10^{-6}. The dilutions were loaded onto MDCK cell monolayers in a 96 well plate (100 µl per well, 4 repeats). After 1 hr absorption, the dilutions were removed, and cells incubated and the viral infection rate determined as described above.

Prevention test

Different EC16 formulations (100 µl) were incubated with MDCK cells for 1 hr in a cell culture incubator, followed by formulation removal and washing with MEM serum-free medium. A series dilution of H1N1 virus in MEM serum-free medium was added to confluent monolayers of MDCK cells, and incubated for 1 h. As described above, the medium was changed and TCID50 infection rate determined after 4 days of incubation.

Treatment test

To test if formulations containing EC16 had a treatment (post-infection) effect, MDCK cells in 96 well cell culture plate were initially infected for one hour with H1N1 virus in series dilutions. Then, 100 µl of formulations containing EC16 were applied to each well onto the cells for one hour before being washed away with MEM serum-free medium. The TCID50 was determined as described above.

Thin layer coating test

To test if a thin layer of formulations containing EC16 applied on top of a cell monolayer could reduce H1N1 infection, 10 µl of formulations containing EC16 was applied to each well (0.3 cm² in area) of a 96-well plate of MDCK cells for either 10 or 30 min. Then, the cells were exposed for 1 hr to an H1N1 challenge in series dilutions without removal of the formulation. The viral dilutions were removed and 200 µl fresh MEM serum-free medium with 0.2 µg/ml trypsin added, and TCID50 determined as above.

Cell viability (MTT) assay

This experiment tested if EC16 formulations were associated with cytotoxicity in MDCK cells. MDCK cells were cultured in a 96 well plate until confluent. MEM serum free medium, MEM medium with 10% carrier and 20% carrier (carrier controls), or MEM medium containing 0.1% EC16 and 10% or 20% carrier was added to the wells followed by a 1 hr incubation at 35°C with 5% CO_2. The medium was then changed to 200 µl fresh MEM serum free medium with 0.2 µg/ml trypsin in each well and incubated overnight under the same condition. The plate was removed from the cell culture incubator and an MTT assay was performed according to a method described previously [21].

All experiments were repeated at least three times.

Statistical analysis

For statistical comparisons, all results were expressed as the percentage treated/untreated virus titer, and percentage values were logit transformed ($y=\ln(x/(100-x))$). Normality was assumed. Within an experimental repeat, transformed values for different treatments were evaluated initially as repeat measures, and groups were compared by one-way or two-way ANOVA (with Geisser-Greenhouse epsilon correction), or t-tests, as appropriate, using Prism v6.0g (GraphPad Software, La Jolla, CA). Where matching was statistically ineffective, ordinary ANOVA was performed. Alpha was set to 0.05. Single groups were tested for a difference to 99.99% or 0.01% (logit values of 6.907 and -9.210) by a one-sample t-test with a Bonferroni correction to alpha of 0.025, 0.017 or 0.013 for respectively 2, 3 or 4 multiple comparisons.

Results

Pilot experiments showed no antiviral activity for 10% or 20% carrier alone in MEM serum-free medium (data not shown; p>0.4).

Viral inactivation test

This experiment was designed to determine whether EC16 was capable of inactivating H1N1 virus rapidly by direct contact for 1 min after mixing with the virus. Figure 1 shows that EC16 at 0.01% in a formulation containing 10% carrier reduced H1N1 infectivity to 20.5% ± 17.1 of control, (n=3) while 0.1% EC16 reduced infectivity to 6.1% ± 3.0. For 0.1% EC16 in 20% carrier, the value was 2.4% ± 1.1. Matching was not effective (p=0.6; n=3, logit transformed values). An additional three separate replicate experiments testing 0.1% EC16 in 10% carrier showed consistent results (3.8% ± 1.5; overall mean (n=6) 5.0% ± 2.4 of control), and in separate experiments testing 0.01% EC16 in 20% carrier, infectivity was reduced to 7.3% ± 9.2% of control.
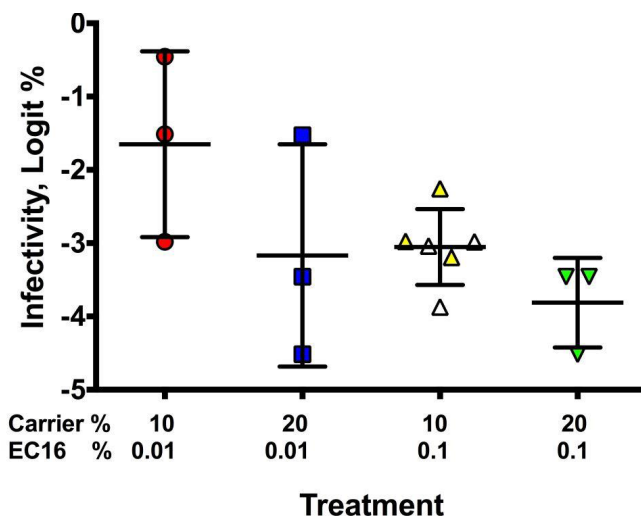

Figure 1: Viral Inactivation test of H1N1 exposure by direct contact with EC16. EC16 was tested at two concentrations (0.01 and 0.1%) in two concentrations of carrier (10% and 20%) by mixing with H1N1 virus for 1 min before MDCK infection and TCID50 determination. Three replicate experiments were performed using 0.01% and 0.1% EC16 in 10% carrier and 0.1% EC16 in 20% carrier; 0.01% EC16 in 20% carrier was tested separately, and three additional repeat tests (open symbols) were performed for 0.1% EC16 in 10% carrier. Mean logit transformed percentage values (large bar) and standard deviations (small bars) are shown.

Ordinary two-way ANOVA using all values showed no significant interaction (p=0.48), and no significant effect for EC16 concentration (p=0.07), but a borderline significant effect for carrier concentration (p=0.048). (However, with the different group sizes and low n, these p values should be viewed with caution.) The main trend was thus for a reduced standard deviation at 0.1% EC16, suggesting a more consistent treatment effect. All four test groups gave significantly less than 99.9% viral activity (one-sample t-test, p ≤ 0.008), and all but 0.01% EC16, 20% carrier (p=0.020; not significant after Bonferroni correction n=4) were significantly higher than 0.01% viral activity (p<0.004). That is, the reduction in activity was significant, but broadly, it remained

significantly above 0% viral activity.

Prevention test

To test the ability of EC16 to prevent cell infection by H1N1, MDCK cell monolayers were incubated with EC16 treatments for 1 hr, and then free EC16 was washed away before cells were exposed to virus. Two sets of experiments were performed: the first compared the effects of 0.01% EC16 10% carrier, 0.1% EC16 10% carrier, and 0.1% EC16 20% carrier (n=4); the second compared 0.01% and 0.1% EC16 in 20% carrier (n=3). At 10% carrier, 0.01% EC16 showed no effect on viral titer (100% viability, n=4; data not shown). There was no significant matching effect in either experiment (p ≥ 0.34), and the mean viral titers from the two sets of experiments using 0.1% EC16 20% carrier also did not differ significantly (unpaired t-test; p=0.08). Therefore, results from the two experiments were combined for analysis (Figure 2). The values (n=7) for 0.1% EC16, 20% carrier were normally distributed (Shapiro-Wilk test, p=0.06).
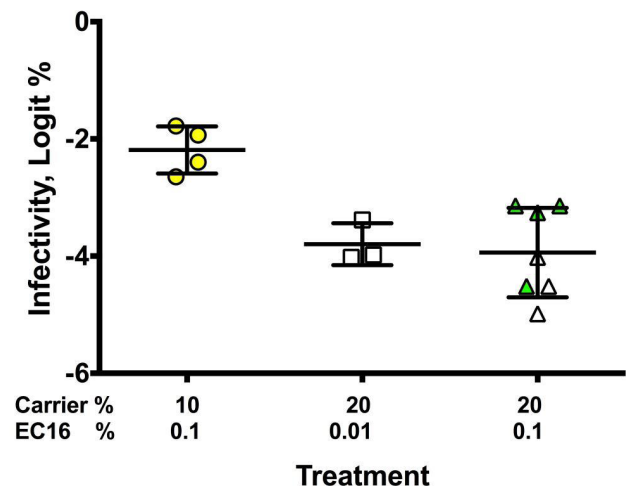

Figure 2: Effect of EC16 pre-treatment of MDCK cells on viral titer (prevention effect). MDCK cell monolayers were incubated with EC16 treatments for 1 h, free EC16 was then washed away, and cells exposed to virus for one hour followed by TCID50 assay. Combined data from two sets of experiments are shown, one comparing 0.1% EC16 at different carrier concentrations (solid symbols) and one comparing different EC16 concentrations at 20% carrier. Mean logit transformed percentage values (large bar) and standard deviations (small bars) are shown for combined data.

All three of these treatments (0.1% EC16 in 10 and 20% carrier, 0.01% EC16 in 20% carrier) gave infectivity values significantly less than 99.9% of control (p<0.004), but greater than 0.01% (p<0.002); the means (± SD) for 0.1% EC16, 10% carrier, and 0.01 and 0.1% EC16 in 20% carrier were respectively 10.5% ± 3.6, 2.3% ± 0.9, and 2.4% ± 1.6.. Ordinary one-way ANOVA of logit transformed data showed a significant difference between the groups (p=0.003), with the mean for 0.1% EC16, 10% carrier being significantly higher compared to the other groups (p<0.015), consistent with the effect of carrier concentration seen in the suspension test. The standard deviations did not differ significantly (Brown-Forsythe test, p=0.11).

To further examine the role of carrier in the EC16 reduction of viral titer, we used 2% dimethyl sulfoxide (DMSO) as an EC16 solvent for the prevention test with different concentrations of EC16 instead of carrier. EC16 at concentrations from 0 to 0.1% in DMSO did not result in a statistically significant difference in comparison to the control viral titer (data not shown). This result indicated that EC16 required a significant amount of organic carrier content (a proprietary polyol compound carrier) in the formulation in order to show effective antiviral activity.

Collectively, these results showed that EC16 in the presence of carrier could substantially reduce MDCK infection by H1N1 virus, and that 0.1% EC16 in 20% carrier gave the greatest (42-fold) reduction in titer.

Treatment test

To determine if EC16 was capable of reducing viral reproduction in MDCK cells that had just been infected with virus, monolayers of MDCK cells were infected with a series of dilutions of H1N1 for 1 hr, then EC16 was applied at either 0.01% in 10% carrier, or 0.01 or 0.1% in 20% carrier. All treatment values were significantly lower than controls (99.99%, p<0.005), indicating an antiviral effect, but significantly higher than 0.01% of control (p<0.008), consistent with some remaining active virus. Figure 3 demonstrates that 0.01% EC16 in 10% carrier was relatively poor and somewhat inconsistent at treating infected cells (viral TCID50 reduced to 15.4% ± 15.2 of control).
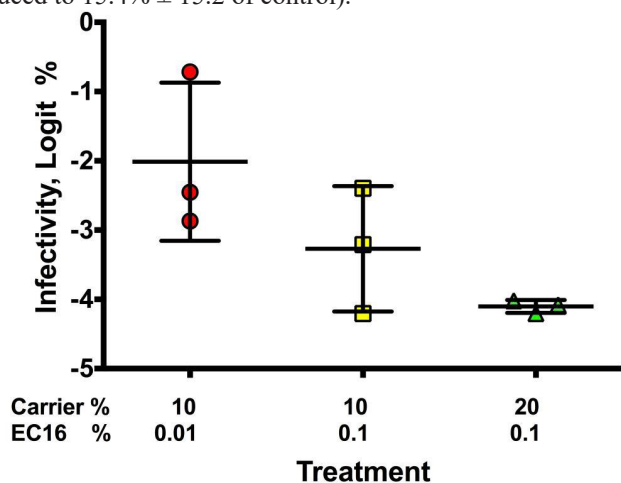

Figure 3: Effect of EC16 formulations on viral reproduction in MDCK cells infected with virus (treatment effect). Monolayers of MDCK cells were infected with a series of dilutions of H1N1 for 1 h, then EC16 formulations were applied for 1 hr prior to TCID50 assay. Mean logit transformed percentage values (large bar) and standard deviations (small bars) are shown.

However, at 0.1%, EC16 in 10% or 20% carrier reduced the viral titer to respectively 4.6% ± 3.5 and 1.6% ± 0.2 of control. Matching was not significant (p=0.3), and the standard deviations did not differ significantly (Brown-Forsythe test, p=0.42). Analysis of the three groups by ordinary one-way ANOVA showed no significant difference between the three treatments (p=0.057; n=3).

Thin layer coating test

These experiments were designed to determine whether a thin layer of EC16 formulation coating the monolayer of MDCK cells (33 µl/cm2 well areas) prevented subsequent H1N1 viral infection. In the first set of experiments, cells were treated for 10 min with 0.01 and 0.1% EC16 in 10% carrier, and 0.1% EC16 in 20% carrier for 10 and 30 min. In the second set of experiments, 0.01 and 0.1% EC16 in 20% carrier were compared at 30 min of treatment (Figure 4). Matching was not effective (p ≥ 0.4) and there was no significant difference between the two sets of data for a 30 min treatment with 0.1% EC16 in 20% carrier (unpaired t-test, p=0.94). Therefore, the two sets of data were combined for analysis.
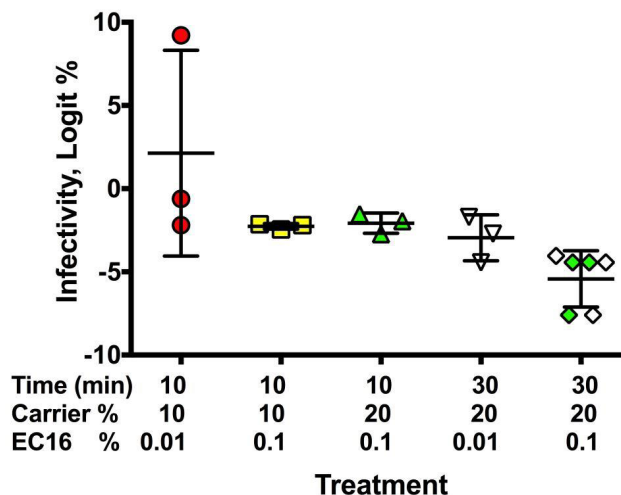

Figure 4: Effect of a thin layer of EC16 formulation coating MDCK cells on subsequent H1N1 viral infection (thin layer prevention effect). Mean logit transformed percentage values (large bar) and standard deviations (small bars) are shown.

EC16 at 0.01% with 10% carrier for 10 min gave inconsistent and poor viral inhibition (remaining viability 48.4% ± 46.4), with remaining infectivity ranging from 10% to 100%. This group was therefore excluded from subsequent analysis. The 10 min treatment groups with 0.1% EC16 in 10 and 20% carrier, and the 30 min treatment groups with 0.01 and 0.1% EC16 in 20% carrier, gave mean values of respectively 9.5% ± 1.4, 12.1% ± 5.8, 7.6% ± 7.2, and 0.9% ± 0.7. These were all significantly less than 99.9% (one-sample t-test, p ≤ 0.007), but (with the exception of 0.01% EC16, 20% carrier, 30 min, p=0.016, not significant after Bonferroni correction, n=4), significantly greater than 0.01% (p ≤ 0.003). For the two 10 min 0.1% EC16 treatments, there was no significant difference in viral titer between 10 and 20% carrier (unpaired t-test with Welch's correction, p=0.65).

Similarly, there was no significant difference between 0.01 and 0.1% EC16 with a 30 min treatment in 20% carrier. However, when 10 min versus 30 min treatments with 0.1% EC16 in 20 carrier were compared, a 30 min treatment gave a significantly greater reduction in titer (p=0.004). Similar results were obtained analyzing the experiments separately.

Cell viability assay

This experiment was designed to determine if EC16 induced cytotoxicity in MDCK cells. Repeat measures one-way ANOVA showed a significant effect in treatment groups (p<0.0001; matching was effective (p=0.033); Geisser-Greenhouse epsilon 0.638). As shown in Figure 5, there was a significant decrease in cell viability induced by 1 hr incubation with 20% carrier alone in comparison to all four treatments (p<0.0005; a 29% reduction in MTT value in comparison to MEM alone, 1.04 ± 0.15 vs. 1.47 ± 0.17 OD units). However, 10% carrier, and EC16 containing formulations with either 10% or 20% carrier, were not statistically different from the MEM control (p>0.6). That is, EC16 protected the cells from the cell viability reduction (or a reduction in metabolic rate as determined by the MTT assay) associated with a high carrier concentration.
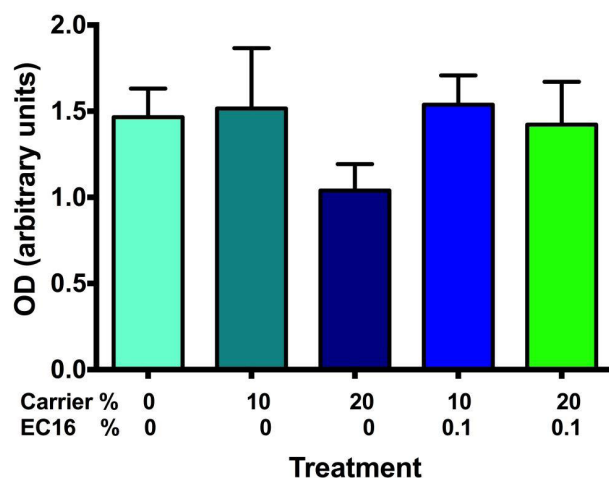

Figure 5: Evaluation of formulation cytotoxic effects by MTT assay. Mean values (n=16) and standard deviations (error bars) are shown.

Discussion

In 2014, based on a systemic review of clinical studies and regulatory comments, a group of international scientists reached a conclusion against the use of oseltamivir (Tamiflu) in clinical practice as an anti-influenza drug, and against WHO's list of this drug as an essential drug for anti-influenza use [22]. Indeed, in 2017 WHO subsequently downgraded oseltamivir to a complementary drug (WHO Executive Summary, The Selection and Use of Essential Medicines, 2017). Unfortunately, according to newly published clinical trial data a new drug immediately in the clinical pipeline, Xofluza, did not demonstrate better efficacy in comparison to oseltamivir [5]. Thus, a new generation of medications with completely different mechanisms of action against a broad-spectrum of influenza virus is in urgent need to better combat flu associated symptoms. A reasonably effective agent would reduce viral titer by at least 10-fold, whilst a good agent would reduce the titer 100-fold [23].

It is known that water-soluble EGCG inactivates enveloped viruses, including influenza virus, effectively, but not certain non-enveloped viruses such as poliovirus — one of the most difficult to eradicate viruses [24]. However, previously studies demonstrated that without direct contact with virus, either pretreatment or post-treatment of cells with water-soluble EGCG had little or no effect on viral infectivity [24], suggesting that EGCG may not be effective in prevention and treatment of flu-associated symptoms, especially for protection of the airway epithelial cells. Indeed, a well-controlled animal study confirmed that an EGCG-containing nasal application failed to protect mice from H1N1-induced death, when either pre-applied or post-applied in the nasal cavity, while the animals survived when the application was mixed with the virus prior to infection [25]. Interestingly, pretreatment with oseltamivir (Tamiflu) 10 mg/kg/day also failed to prevent any animal death [25]. Another study using a ferret model of influenza infection similarly showed that an EGCG-containing nasal application with horseradish did not effectively prevent viral infection, even when applied only 5 min after nasal delivery of H3N2 virus, which requires much longer than 5 min for absorption [26]. These results indicate that water-soluble EGCG or green tea extract containing a large portion of EGCG are not effective in prevention of influenza viral infections unless in direct contact with the virus. In addition, the poor stability of nasal formulations with water-soluble EGCG is another major challenge to their clinical use.

In contrast to these results, our group has reported that EC16, a stable lipophilic EGCG form, is able to effectively inactivate both poliovirus and also norovirus, which is a non-enveloped virus that is resistant to alcohol [27,28]. We also showed in previous work that lipophilic EGCG is highly active against herpes simplex virus 1 [17]. Similarly, lipophilic EGCG has been reported to be active against influenza viruses [16]. This evidence suggests that EC16 possesses higher efficacy to inactivate a broader spectrum of pathogenic viruses than water-soluble EGCG. Taken together, modification of EGCG into a highly effective, more stable, and lipid-soluble derivative could provide an ideal candidate for a prophylactic and therapeutic agent against seasonal flu [29].

We hypothesize that lipophilic epigallocatechin-3-gallate (L-EGCG), EGCG-palmitate (EC16) in particular, is able to fill the gap in effective methods to combat seasonal flu, and become a first-in-class prophylactic and therapeutic agent against influenza virus infection. As an initial test of our hypothesis, this exploratory investigation first examined the efficacy of EC16 in viral titer reduction when it was mixed with H1N1 virus for only 60 sec. As shown in Figure 1, infectivity of H1N1 was inhibited by approximately 98% using 0.1% EC16 formulated with 20% carrier in MEM medium. Information gained from this experiment indicated that higher carrier content could enhance inhibition, and suggested a higher EC16 concentration gave more consistent inhibition. As a lipid-soluble molecule, EC16 is not soluble in aqueous cell culture medium and therefore organic materials as carriers play a key role in maintaining EC16 in aqueous solution, and therefore in activity.

Also, when EC16 is associated with organic molecules instead of water, the stability of EC16 is enhanced due to the decreased exposure to oxygen, and additionally, the binding to viral protein could be further strengthened. Although it was known that direct contact of EGCG or L-EGCG with influenza virus results in an

inhibitory effect, the present results show that this contact need only last for 60 sec. This rapid inactivation of viral infectivity when exposed to EC16 for 60 sec has been shown previously for the most difficult to inactivate and alcohol-resistant nonenveloped viruses, such as poliovirus and norovirus [27,28].

Unlike previously reported studies using water-soluble EGCG [19], exposure of MDCK cells to EC16 at 0.1% with 20% carrier for 1 hr before washing and medium change effectively prevented (by almost 99%) subsequent H1N1 infectivity (Figure 2). This effect of EC16 or EGCG has not been reported previously. Carrier content in the formulation is required for this effect because the identical concentration of EC16 with 2% DMSO failed to deliver similar outcomes (data not shown). These results suggested that EC16 incubation with MDCK cells leads to a "coating" effect to the cells, possibly through the insertion of the fatty acyl chain into the hydrophobic portion of cell membrane, thereby retaining EGCG to inactivate H1N1 virus when the virus subsequently encounters the cell membrane. Another potential mechanism is the binding of EC16 to cell surface sialic acid-containing glycoproteins, thereby preventing the binding and internalization of H1N1 into the cells. However, the exact mechanisms require further exploration. This efficacy of a formulation with EC16 suggests a novel concept of a "barrier for prevention" that can be explored, with the potential to provide a breakthrough in flu prevention by further improve the formulations to maximize the efficacy.

EC16 has been demonstrated to have superior efficacy against influenza viruses in comparison to water-soluble EGCG and to other antiviral drugs. Kaihatsu et al. reported in 2009, using H5N2 virus in a chicken embryo infection system, that EC16 was the only agent that protected 100% of chicken embryos, while EGCG was the second best, protecting >30% embryos. Oseltamivir was the least effective agent tested, with less than 20% of embryos surviving, while Zanamivir protected less than 30% of embryos [30]. In that study, it was also demonstrated that EC16 effectively inhibited H1N1, H3N2 and influenza B viruses [30].

In the present study, to test the treatment effect of EC16, MDCK cells were initially infected by H1N1 virus absorption for 1 h, and then the cells were incubated with EC16 with carrier for 1 hr before a medium change and TCID50 assay. Treatment with 0.1% EC16 with 20% carrier led to a >98% decrease in infectivity of H1N1 virus (Figure 3). This post-infection treatment result suggested that EC16 could be an effective agent for therapeutic applications to reduce symptoms in the respiratory tract caused by influenza virus.

Another concept tested in the current study was whether a thin layer of EC16 coated on a cell surface could effectively prevent H1N1 virus infection. The experiments were designed to allow the thin layer to cover the cell surface for 10 min or 30 min followed by H1N1 virus infection (1 hr absorption). Figure 4 shows that a 10 min thin layer application resulted in an 88% reduction in viral titer with 0.1% EC16 and 20% carrier. When the thin layer coverage was increased to 30 min, infection was reduced by about 99%. This result indicated that topical application in the nasal or oral cavity has the potential to effectively reduce viral infection of the epithelial cells, pending additional formulation and efficacy studies with various influenza viruses. A foreseeable challenge is how to coat the cell surface on the respiratory tract for an extended period as a barrier for prevention, which would need to be addressed in animal models.

EC16 belongs to a group of fatty acyl esterified EGCG derivatives with altered physical and chemical properties in comparison to water-soluble EGCG. The chemical modification of EGCG appears to enhance the safety of the compounds in comparison to natural EGCG under the intended use range, as tested previously in laboratory animals [31,32]. The EC16-rich "tea polyphenol-palmitate" has been approved for use as a food item [29]. The cell viability test results here demonstrated that EC16 formulated with carrier did not induce cytotoxicity in MDCK cells (Figure 5). Interestingly, 20% carrier decreased cell viability (or at least, MTT-measured metabolic activity) significantly in comparison to the untreated control, but 0.1% EC16 reversed this carrier effect (Figure 5). It is possible that excessive carrier interferes with cell membrane integrity or metabolism in cell culture settings, and EC16 protected the cells from these effects [33]. Therefore, future formulation of EC16 for human use may be combined with other carriers such as essential oils and mineral oil to further enhance the activity and stability.

Here we report, for the first time, that coating the cell surface with EC16 in proprietary carrier-containing formulations substantially blocked H1N1 virus infectivity, without direct contact with the free virus, and EC16 effectively treated H1N1-infected cells after a single application. To the best of our knowledge, these novel observations have not been reported previously. These results contradicted previously established dogma that the anti-influenza effects of (water-soluble) EGCG depend on direct contact of the virus, and it is not useful for prophylactic and therapeutic purposes if applied before or after influenza virus infection. In conclusion, the chemically modified EGCG, EC16, demonstrated significant potential for a new generation of a multi-targeted anti-influenza agent with a wide-spectrum of action, high efficacy, stability, and without a risk of developing drug-resistant variant strains. Based on the data generated from this study, further investigation is warranted into determining the suitability of using EC16 against infections from pathogenic viruses such as influenza using different models and formulations, in order to develop a new generation of medications to protect human population from virus-associated epidemics and pandemics or biological warfare. With further investigation of additional influenza viruses such as H3N2 and influenza B subfamily members, and improvements in the formulation, EC16 could be a suitable candidate for a first-in-class nasal/mucosal application to prevent influenza infection by protecting the epithelial cells in the respiratory tract.

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