Inhibition of influenza virus infection by epigallocatechin-3-*O*-gallate (EGCG) fatty acid monoesters

Kunihiro Kaihatsu

Dept. of Organic Fine Chemicals, The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan

Correspondent email: kunihiro@sanken.osaka-u.ac.jp

Summary

A series of EGCG-fatty acid derivatives were synthesized by lipase-catalyzed transesterification. EGCG-monoesters with long alkyl chains efficiently inhibited human-pathogenic influenza A and B viruses, including clinically isolated oseltamivir phosphate-resistant and amantadin-resistant viruses, in Madin-Darby canine kidney cells. Further, EGCG-monopalmitate strongly inhibited avian influenza A virus in chicken embryos. Transmission electron microscopy of influenza A/Puerto Rico/8/34(H1N1) virions showed that EGCG-monopalmitate induced morphological changes in the viral surface structure more efficiently than EGCG or detergents such as sorbitane monopalmitate. EGCG-monopalmitate blocked virus infection at an early stage. These results indicate that EGCG-monopalmitate interacts with the viral proteins responsible for viral attachment to, or membrane fusion with, the host cell.

Introduction [Please cite Figure 1 in the text.]

Seasonal influenza epidemics and pandemics cause significant disease burdens and mortality in humans. Surprisingly, there are only a few prescribed antiviral drugs for the treatment and prophylaxis of influenza. A neuraminidase inhibitor, oseltamivir phosphate, is the most commonly used antiviral drug, and acts by preventing the release and budding of viral particles from infected cells. However, it has been reported that current seasonal influenza viruses, and a highly pathogenic avian influenza (H5N1), are resistant to oseltamivir (de Jong, *et al*. 2005). Moreover, the limited availability of the drug's starting material, shikimic acid, has lead to shortages in the drug's supply. Therefore, there is an urgent need to develop a novel anti-influenza virus agent.

(–)-Epigallocatechin-3-*O*-gallate (EGCG; **1**), a major component of green tea, has antiviral activity (Nakayama, *et al.* 1993; Imanishi, *et al.* 2002; Yamaguchi, *et al.* 2002; Song, *et al.* 2005). Studies on the anti-influenza activity of **1** found it inhibits virus adsorption (Nakayama, *et al.* 1993), as well as acidification of endosomes and lysosomes (Imanishi, *et al.* 2002). This inhibitory effect is different from other current neuroaminidase (NA) or proton pump inhibitors, suggesting that **1** is a new class of virus inhibitory compound effective against drug resistant influenza strains. Unlike the starting material for most other drugs, good quality EGCG can be easily obtained from tea extract at a reasonable cost. Therefore, there are compelling reasons for developing antiviral compounds from **1** so that patients infected with influenza virus can be effectively treated.

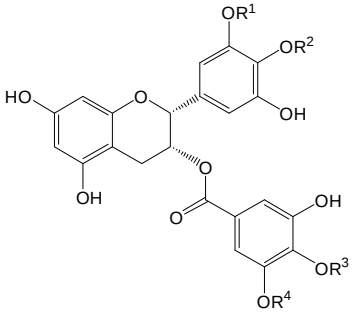

Fig. 1. Chemical structure of EGCG (**1**) and EGCG-C16 (**2**). **1**: R^1, R^2, R^3, and R^4 = H, **2**: one of R^1, R^2, R^3, or R^4 = $-CO(CH_2)_{14}CH_3$; others = H. The ratio of the acyl position at R^1:R^2:R^3:R^4 = 38:35:7:20.

Currently, **1** is not used as an antiviral compound because of its poor lipid membrane permeability (Tanaka, *et al*. 1998) and poor stability (Hong, *et al.* 2002). Several strategies have been pursued to improve the biological properties of **1**. The introduction of alkyl groups increases the lipid membrane permeability of **1** (Tanaka, *et al*. 1998), while peracetylation increases its stability under physiological conditions (Lam, *et al*. 2004). Recently, we reported a method for the synthesis of EGCG-fatty acid monoesters using lipase-catalyzed transesterification (Mori, *et al*. 2008). We also confirmed that the

inhibitory effect of EGCG-fatty acids on influenza virus increased as the alkylation length increased. EGCG-monopalmitate (**2**) showed broad spectrum activity against human and avian pathogenic influenza viruses, including drug resistant strains, with an antiviral activity 44-fold higher compared to EGCG (Kaihatsu, *et al*. 2009). Compound **2** completely inhibited avian influenza virus infection in chicken embryos, while **1** and other prescribed drugs did not. Here we report the antiviral mechanism of action of **2** as investigated by a plaque formation reduction assay, a neuraminidase inhibition assay, fluorescence immunostaining, and transmission electron microscopy (TEM).

Materials and methods

Measurement of direct influenza virus inhibition activity

The direct anti-influenza virus activities of **1**, **2**, a prescribed anti-influenza drug (zanamivir), and detergents (n-dodecyl-β-D-maltoside and sorbitan-monopalmitate) were evaluated by a plaque formation assay. Each compound at different concentrations in dimethyl sulfoxide (DMSO) was mixed with influenza virus in Opti-modified Eagle's medium (Opti-MEM) containing 0.2% DMSO. After incubating for 30 min at room temperature, the mixture was applied to a confluent monolayer of Madin-Darby canine kidney (MDCK) cells in a 6-well plate (multiplicity of infection, MOI = 0.0033). The influenza virus inhibition activity of the compounds was assessed by plaque reduction on the MDCK cell monolayer.

Neuraminidase (NA) inhibition assay

The NA inhibitory activity of each compound was evaluated using fluorescently labeled sialic acid as a substrate (Nayak, *et al*. 2004). A 50-μL aliquot of each compound in Opti-MEM containing 0.2% DMSO at different concentrations was mixed with 50 μL of 2×10^4 $TCID_{50}$ (50% tissue culture infective dose) /mL of influenza virus A/Puerto Rico/8/34(H1N1) in 50 mM sodium phosphate buffer containing 0.2% DMSO. These virus/antiviral compound solutions were incubated for 30 min at room temperature. An equal volume of 250 μM 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid was added and the mixture was incubated for 1 h at 37 °C. After adding 800 μL of stop solution (25% ethanol, 0.1M glycine, pH10.7), fluorescence from degraded 4-methylumbelliferone was measured by fluorescence spectrometry (excitation 365 nm, emission 448 nm).

Detection of hemagglutinine (HA) protein production in influenza virus-infected MDCK cells

Compound **2** was incubated with influenza virus A/Puerto Rico/8/34(H1N1) in Opti-MEM containing 0.2% DMSO for 30 min at room temperature. The mixture was then applied to a confluent monolayer of MDCK cells in a 6-well plate (MOI = 0.03) and further incubated for 1 h at room temperature to allow the virus particles to adhere. The solution was removed and the cell sheets were washed twice with D-PBS, and maintained in D-MEM containing 0.2% BSA. After incubating for 6, 8, or 10 h, the cell sheets were immobilized using methanol and stained with anti-HA antibody and Texas red-labeled anti-mouse IgG. The nuclei were stained with Hoechst 33258. The viral HA protein was detected by fluorescence microscopy.

Electron microscopy.

To study the morphology of influenza virus A/Puerto Rico/8/34(H1N1) in the presence of **2**, the virus was incubated with various concentrations of **2** for 30 min. In order to negatively stain influenza virus, sucrose gradient-purified influenza virus was applied to a carbon-coated collodion grid for 30 sec. Excess influenza virus solution was removed by blotting with filter paper, and then the grid was immediately stained with 1% phosphotungstic acid for 30 sec. Excess stain was removed with filter paper and the samples were examined using a transmission electron microscope.

Results and discussion

We previously reported that **1** and **2** inhibit a broad spectrum of influenza A and B viruses, including drug resistant strains (Kaihatsu, *et al.* 2009). Compound **2** exhibited a potent antiviral effect, since it was directly incubated with the viruses, although its antiviral mechanism of action remained unclear.

Therefore, we prepared several compounds, listed in Table 1, and evaluated their direct antiviral activities. Compound **2** showed 20-fold and 2,000-fold higher antiviral activity compared to **1** and sorbitan-monopalmitate, respectively. The introduction of a palmitoyl group on EGCG enhanced anti-influenza virus activity, probably because of the compound's increased affinity for the viral membrane. The EGCG backbone is thought to be important for inactivating virus infectivity because sorbitane-monopalmitate showed much weaker direct virus inhibition activity. Furthermore, zanamivir, a prescribed neuraminidase inhibitor, showed poor direct virus inhibition activity. These results indicate that the main target of **1** and **2** is neither the viral membrane nor the NA protein. Indeed, **1** and **2** showed much weaker NA inhibition activities compared to zanamivir (Table 1).

Table 1. Comparison of direct virus inhibitory and neuraminidase inhibitory effects of virus inhibitory compounds.

Compounds	Direct virus inhibition activity EC_{50} (μM)	NA inhibition activity EC_{50} (μM)
1	0.391±0.056	50.0
2	0.020±0.007	0.600
Zanamivir	9.64	0.002
n-Dodecyl β-D-maltoside	25.0	ND
Sorbitan-monopalmitate	40.0	ND

To study the antiviral mechanism of action of **2**, we monitored viral protein production in influenza virus-infected MDCK cells by fluorescence immunostaining. Compound **2** inhibited early stage viral infection (Fig. 2). Moreover, **2** inhibited adsorption between influenza viruses and chicken red blood cells (data not shown). From these results, we conclude that **1** and **2** likely interact with viral surface components, such as HA protein, and inhibit the infection. This conclusion is in accord with current reports (Nakayama, *et al*. 1993; Song, *et al*. 2007).

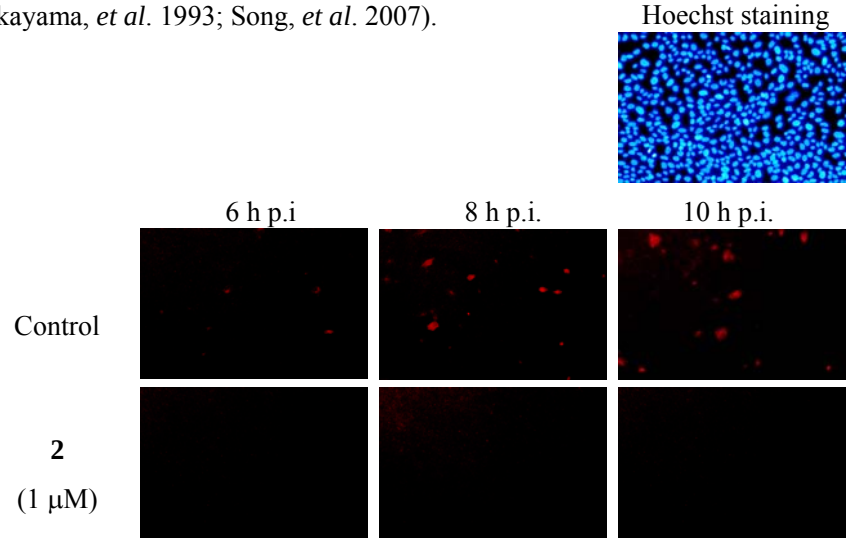

Fig. 2. Effect of **2** on HA protein expression of influenza virus-infected MDCK cells. HA protein was monitored at 6, 8, and 10 h post infection (p.i.) using anti-HA antibody and Texas red-labeled secondary antibody. Nuclei of the cells were stained with Hoechst 33258.

To study how **2** inactivates virus infectivity, we analyzed the morphological changes in influenza A/Puerto Rico/8/34(H1N1) in the presence of **2** by transmission electron microscopy, and found that **2** induces conformational changes in the viral surface proteins in a dose-dependent manner (Fig. 3).

Taking these results together, **2** may interact with influenza virus membrane proteins, such as HA protein, and induce conformational changes that render the virus incapable of infecting cells.

Our newly synthesized EGCG-monopalmitate, **2**, may represent a new class of antiviral agent that directly inhibits the infectivity of influenza viruses.

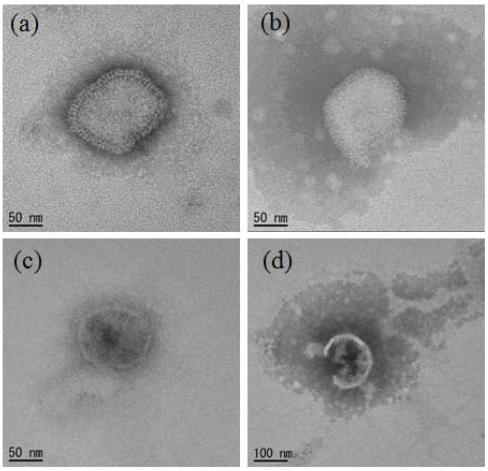

Fig.3 Negative staining of a purified influenza virion (influenza A/PR8/34(H1N1)) in the presence of (a) 0 nM, (b) 2 nM, (c) 20 nM, and (d) 500 nM of **2**

Acknowledgements

This study was supported by the Industrial Technology Research Grant Program in 2006 from New Energy and Industrial Technology Development Organization (NEDO) of Japan. We thank Taiyo Kagaku Co. Ltd. for their kind donation of EGCG.

References

1. de Jong, MD., Tran, TT., Truong ,HK. et al. (2005) Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 353:2667-2672
2. Nakayama, M., Suzuki, K., Toda, M. et al. (1993) Inhibition of the infectivity of influenza virus by tea polyphenols. Antiviral Res. 21:289-299
3. Imanishi, N., Tuji, Y., Katada, Y. et al. (2002) Additional inhibitory effect of tea extract of the growth of influenza A and B viruses in MDCK cells. Microbiol Immunol. 46:491-494
4. Tanaka, T., Kusano, R., Kouno, I. (1998) Synthesis and antioxidant activity of novel amphipathic derivatives of tea polyphenol. Bioorg Med Chem Lett. 8:1801-1806
5. Hong, J., Lu, H., Meng, X. et al. (2002) Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (–)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. Cancer Res. 62:7241-7246
6. Lam, WH., Kazi, A., Kuhn, DJ. et al. (2004) A potential prodrug for a green tea polyphenol proteasome inhibitor: evaluation of the peracetate ester of (–)-epigallocatechin gallate [(–)-EGCG]. Bioorg Med Chem. 12:5587-5593
7. Mori, S., Miyake, S., Kobe, T. et al. (2008) Enhanced anti-influenza A virus activity of (–)-epigallocatechin-3-O-gallate fatty acid monoester derivatives: effects of alkyl chain length. Bioorg Med Chem Lett. 18:4249-4252
8. Kaihatsu, K., Mori, S., Matsumura, H., Daidoji, T., Kawakami, C., Kurata H., Nakaya T., Kato, N., (2009) Broad and potent anti-influenza virus spectrum of epigallocatechin-3-*O*-gallate -monopalmitate. J. Mol. Genet. Med. 3:195-197
9. Nayak, D.P., Reichl, U., (2004) Neuraminidase activity assays for monitoring MDCK cell culture derived influenza virus. J. Virol Meth., 122: 9-15
10. Song, JM., Park, KD., Lee, KH. et al. (2007). Biological evaluation of anti-influenza viral activity of semi-synthetic catechin derivatives. Antiviral Res, 76:178-185