LETTER

Broad and potent anti-influenza virus spectrum of epigallocatechin-3-*O*-gallate-monopalmitate

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Numerous influenza pandemics in the last century resulted in the death of millions of people worldwide. With the current and future threats of influenza pandemics development of new antiviral compounds remains a great demand. However, currently only a limited number of drugs are available for the treatment and prophylaxis of influenza. A neuraminidase (NA) inhibitor, oseltamivir phosphate, is the most commonly used antiviral drug. However, a number of reports suggest the emergence of oseltamivir phosphate resistance in new seasonal influenza viruses and highly pathogenic avian influenza (H5N1) (for example, de Jong et al, 2005).

(–)-Epigallocatechin-3-*O*-gallate (EGCG: **1**), a major component of green tea plant (*Camellia sinensis*), has been recognized to possess antiviral properties. Reports on the anti-influenza activity of **1** found that it inhibited virus adsorption (Nakayama et al, 1993), as well as acidification of endosomes and lysosomes (Imanishi et al, 2002). Such virus inhibition activity is different from other current NA or proton pump inhibitors, suggesting that **1** can be developed into a new class of antiviral compounds that are effective against current drug resistant influenza strains. However, **1** has not been used as an antiviral compound because of its poor lipid membrane permeability and low chemical stability. It was previously reported that the introduction of long alkyl chain groups to **1** improved its lipid membrane permeability (Tanaka et al, 1998), while protection of its hydroxyl groups with acyl groups increased its chemical stability under physiological conditions (Lam et al, 2004). Recently, we reported a method to synthesize EGCG-fatty acid monoesters using lipase-catalyzed transesterification and demonstrated that EGCG-fatty acids monoesters possessed improved influenza virus inhibitory effect against influenza A/PR8/34/(H1N1) in an alkyl length dependent manner (Mori et al, 2008). Here, we investigated the spectrum of influenza virus inhibition activity of EGCG (**1**) and EGCG-C-16 (**2**). As shown in Figure 1a, **2** is a mixture of four regio-isomers and the ratio of each regio-isomer 2a:2b:2c:2d is 38:35:7:20, respectively. Because the B-ring-modified esters (2a and 2b) and D-ring-modified esters (2c and 2d) showed exactly the same antiviral activities (data not shown), we used the mixture of four regio-isomers (2a-d) in the following assays.

A series of human influenza viruses, an experimental strain (A/Puerto Rico/8/34/(H1N1)), vaccine strains (A/Beijing/262/95/(H1N1), A/Panama/2007/99/(H3N2), and B/Yamanashi/166/98/), drug-resistant strains (Yokohama/77/2008/(H1N1) OPR: oseltamivir phosphate-resistant (OPR), Yokohama/63/2007/(H1N1) AR: amantadine-resistant (AR), A/Yokohama/91/2008/(H1N1) OPR/AR: (OPR/AR) and avian pathogenic influenza (A/Duck/Hong Kong/342/78/(H5N2)), were directly incubated with **1** or **2** for 30min prior to inoculation into MDCK cells. The cells were inoculated with virus, with or without the compounds, for 1hr, and the direct virus inhibitory effects were assessed by a plaque formation assay at 54hr post-incubation. The plaque inhibition activity was calculated relative to no compound. The cytotoxicity of compounds on MDCK cells were evaluated by the MTT cell proliferation assay. Briefly, the cells were incubated with **1** or **2** for 24hr for the MTT assay. The

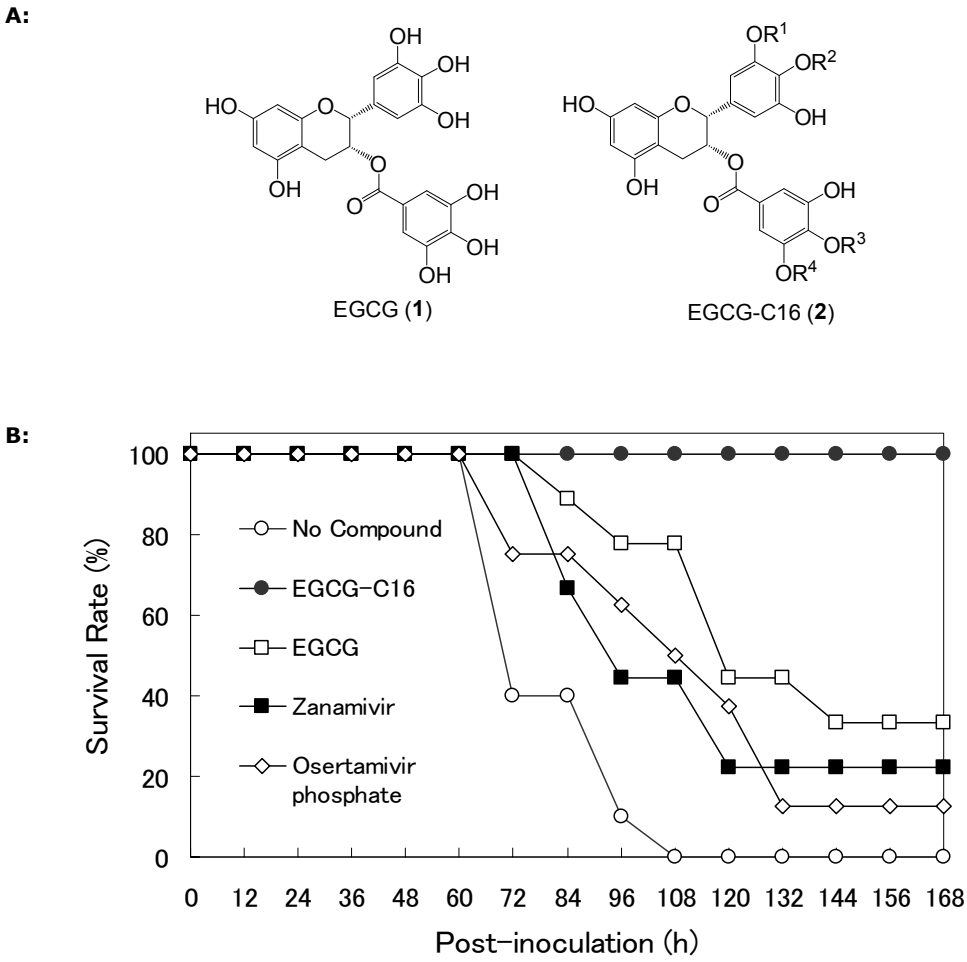

Figure 1. A. Chemical structure of EGCG (**1**) and EGCG-C16 (**2**). **2** is a mixture of four regio-isomers (**2a-d**). **2a**: R^2=R^3= R^4=H, R^1= $CO(CH)_{14}CH_3$, **2b**: R^1=R^3=R^4=H, R^2= $CO(CH)_{14}CH_3$, **2c**: R^1=R^2=R^4=H, R^3= $CO(CH)_{14}CH_3$, **2d**: R^1=R^2=R^3=H, R^4= $CO(CH)_{14}CH_3$. **B**. Avian influenza virus inhibition activity of 1 and 2 in chicken embryonated eggs. Influenza A/Duck/Hong Kong/342/78 (H5N2) was pretreated with or without 1.0μM compounds and then inoculated (50 pfu/egg) into the allantoic fluid of embryonated eggs for 7 days at 37 °C. Open circle, no compound; Closed circle, 1.0μM EGCG-16 (**2**); Open square, 1.0μM EGCG (**1**); Closed square, 1.0μM Zanamivir; Open lozenge, 1.0μM Oseltamivir phosphate.

results of the plaque inhibition assay and MTT assay are expressed as mean ± standard error of three independent experiments.

We also investigated the virus inhibition activity of **1** and **2** on avian influenza A/Duck/Hong Kong/342/78 (H5N2) virus *in ovo* using 11-day-old chicken embryonated eggs (n=12) inoculated with compound-treated or untreated viruses.

As shown in Table 1, both **1** and **2** showed broad virus inhibitory effects on MDCK cells. The EC_{50} values of **2** on these viruses were between 10 to 61nM, which were approximately 7.1 to 44-fold lower than those of **1**. The CC_{50} value of **2** was 82μM, which was only 3.1-fold lower than that of **1** (255 μM). Thus, the SI value of **2** was improved 2.2 to 14-fold compared to **1**.

With respect to the avian influenza virus inhibition assay *in ovo*, virus treated with **1**, zanamivir, or oseltamivir phosphate showed a moderate viral inhibitory effect (Figure 1b). However, **2** almost completely inhibited the infection (Figure. 1b), and the efficacy was retained even at 0.1μM (data not shown).

In summary, **2** inhibited human and avian influenza A and B viruses, including drug-resistant viruses. **2** was found to be more effective than neuraminidase inhibitors, and strongly inhibited the infection of avian influenza (H5N2) virus in chicken embryonated eggs. This unique viral inhibitory action has the potential to be utilized to effectively control a broad spectrum of influenza viruses.

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COMPETING INTERESTS

None declared.

Table 1. Direct virus inhibitory effect of **1** and **2** based on the plaque formation assay

Virus	Direct virus inhibitory effect[a]		SI[b]	
	EC_{50}[c] (μM)		CC_{50}[d] / EC_{50}	
	1	**2**	**1**	**2**
A/Puerto Rico8/34 (H1N1)	0.391±0.056	0.020±0.007	653	4062
A/Beijing/262/95 (H1N1)	0.436±0.081	0.061±0.008	585	1331
A/Yokohama/77/2008 (H1N1) OPR[e]	0.400±0.042	0.017±0.008	638	4779
A/Yokohama/63/2008 (H1N1) AR[f]	0.540±0.042	0.027±0.008	472	3009
A/Yokohama/91/2008 (H1N1) OPR/AR	0.597±0.015	0.036±0.012	427	2256
A/Panama/2007/99 (H3N2)	0.173±0.006	0.010±0.004	1476	8125
A/Duck/Hong Kong/342/78 (H5N2)	0.657±0.022	0.015±0.006	388	5416
B/Yamanashi/166/98	0.490±0.053	0.024±0.008	521	3385

[a]Direct virus inhibitory effect: Virus treated with **1** or **2** for 30min at 37 °C prior to the inoculation and then inoculated for 2 days at 37°C. MDCK cells, MIO: 2.5 x 10^{-4} pfu/cell.
[b]SI (selectivity index) is the ratio of CC_{50} and EG_{50}.
[c]EC_{50} represents the concentration of compound required to reduce plaque number by 50% relative to the control well without test compound.
[d]CC_{50} represents the concentration of compound required to reduce cell viability by 50% relative to the control well without test compound. The CC_{50} for **1** and **2** were 255.7 ± 6.0 and 81.2 ± 12.5μM, respectively.
[e]OPR: oseltamivir phosphate-resistant virus
[f]AR: amantadine-resistant virus

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