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Biological evaluation of anti-influenza viral activity of semi-synthetic catechin derivatives

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Abstract

Catechin derivatives with different alkyl chain length and aromatic ring substitutions at the 3-hydroxyl group were synthesized from epigallocatechin (EGC) and (+)-catechin (C) and their anti-influenza viral activity were evaluated in vitro and in ovo. Pronounced antiviral activity was observed for derivatives carrying moderate chain length (7–9 carbons) as compared to those with aromatic rings, whereas the 5′-hydroxyl group of the trihydroxy benzyl moiety did not significantly contribute to antiviral activity. The derivatives exerted inhibitory effects for all six influenza subtypes tested including three major types of currently circulating human influenza viruses (A/H1N1, A/H3N2 and B type), H2N2 and H9N2 avian influenza virus. The compounds strongly inhibited adsorption of the viruses on red blood cell (RBC). They also restricted the growth of avian influenza virus in ovo with minimum inhibition concentration (MIC) of 5–10 μM far exceeding the neuraminidase (NA) inhibitor oseltamivir or M2 proton channel inhibitor amantadine. The antiviral activity appears to be mediated by interaction with hemagglutinin (HA)/viral membrane rendering HA less fusogenic at the initial stage of infection. The broad spectrum activity against various subtypes of influenza viruses may complement the limitations of current antivirals and contribute for managing potentially emerging influenza pandemic. The structure-activity data of catechin derivatives may usefully guideline future research endeavors for applying green tea catechins as alternative anti-viral agents.

Keywords: Influenza virus; Catechin derivatives; Alkyl chain; Hemagglutinin

1. Introduction

Influenza viruses cause respiratory disease in humans and animals with high morbidity and mortality rates. Spanish flu, the well-known influenza pandemic of 1918, is thought to have killed up to 100 million persons (Reid et al., 2001). Besides the currently circulating H3N2 and H1N1 strains, the H5N1, H7N7, and H9N2 avian influenza viruses are now presenting a potential threat to the human population. The World Health Organization (WHO) recommends the development of pandemic vaccines suitable for mass immunization in addition to the stockpiling of antiviral medicines. Antiviral drugs, such as amantadine and oseltamivir, have been used for the treatment of influenza virus infection. Amantadine and its derivative, rimantadine, block the ion channel function of the M2 protein of the influenza A virus whereas zanamivir and oseltamivir inhibit neuraminidase. These drugs have been proven to be useful for reducing clinical symptoms, but their utility has been limited by side effects and the emergence of resistant viral strains (Gubareva et al., 1998; Kiso et al., 2004; Le et al., 2005). Drugs with increased affinity to neuraminidase are being favorably evaluated as alternatives to oseltamivir (Bantia et al., 2006; Duff and Ashley, 1992).

Previously, several studies have been conducted on catechin derivatives with a view to improve both the physiological and pharmacokinetic properties of the catechins for various applications. The dependence of catechin antibacterial activity on the length of its alkyl side chain at the 3-hydroxyl of the catechin moiety has been observed (Kajiya et al., 2004; Peter, 1987; Peter and Marcia, 1989). Similarly, 3-*O*-acylated (−)-epicatechin and

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epicatechin gallate (ECG), which have varying (C4–C18) chain lengths, have a greater propensity to interact with lipid bilayers, resulting in significant increases in antibacterial activities (Park et al., 2004b; Stapleton et al., 2004). The introduction of an acyl group at the 3-hydroxyl position of (−)-EGC, which improves cell membrane permeability, resulted in a pronounced inhibitory effect on Epstein–Barr virus early antigen activation in Raji cells (Uesato et al., 2003). Catechin and theaflavin digallate derivatives inhibit HIV-1 entry into cells by targeting gp41 and block membrane fusion.(Liu et al., 2005) Moreover, improved chemopreventive activity against tumors has been observed with catechin derivatives (Kumagai et al., 2003; Nam et al., 2001; Park et al., 2004a; Uesato et al., 2000).

Epigallocatechin gallate (EGCG), a major catechin in green tea, is known to affect the infectivity of influenza virus in cell culture. EGCG was shown to agglutinate the viruses and prevent them from absorbing to Madin–Darby canine kidney (MDCK) cells (Nakayama et al., 1993). Recently, structure-function relationships of various catechins (i.e., EGCG, ECG, and EGC) have been investigated for their antiviral activities against various influenza strains. Results showed that the 3-galloyl group of the catechin skeleton plays an important role in its antiviral activity whereas the 5′-OH group in the trihydroxybenzyl moiety at the 2-position has a minor role (Song et al., 2005). The results also showed that substitution or modification of the 3-hydroxyl position significantly affected the antiviral activity. In this study, we synthesized derivatives of (+)-catechin and (−)-epigallocatechin that differ at the 3-hydroxyl position by containing various aromatic or aliphatic alkyl chains. We evaluated their antiviral activities against human and avian influenza viruses.

2. Materials and methods

2.1. Viruses and cells

Avian influenza A/Chicken/Korea/ms96/96 (H9N2) virus, a low pathogenic avian influenza virus that was isolated from a poultry farm in South Korea, was provided by the National Veterinary Research and Quarantine Service. A/Sydney/5/97 (H3N2) virus was provided by the National Institute for Medical Research in London, UK and A/PR/8/34(H1N1), A/Japan/305/57(H2N2), and B/Lee/40 were provided from University of Oxford, UK. MDCK cells were obtained from the American Type Culture Collection (ATCC) and cultured in Minimum Essential Medium (MEM, Invitrogen Co.) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Co.). MDCK cells were used for plaque assay of influenza viruses and for the evaluation of the cytotoxicity of the catechin derivatives.

2.2. Compounds

Based on previous reports (Park et al., 2004a,b) the synthesis of 3-*O*-alkyl-catechin derivatives was modified as described below and also the compounds were confirmed of their structure by HPLC.

2.2.1. Synthesis of 3-O-alkyl-(−)-epigallocatechins

The synthesis of the 3-*O*-alkyl-(−)-epigallocatechins is outlined in Fig. 1. (−)-Epigallocatechin gallate (3 g, 6.54 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF) in a reaction vessel with stirring. Potassium carbonate (K_2CO_3, 10.8 g, 12 eq.) was added to the vessel and the reaction mixture was incubated with 7.4 mL benzyl bromide (8 eq.) for 20 h at room temperature, producing 5,7,3′,4′,5′,3″,4″,5″-octa-*O*-benzyl epigallocatechin with a 87% yield. The 5,7,3′,4′,5′,3″,4″,5″-octa-*O*-benzyl epigallocatechin derivative was treated with NaOH in DMF/H_2O (4:1) for 20 h at 160 °C to produce 5,7,3′,4′,5′-penta-*O*-benzyl-(−)-epigallocatechin with a 94% yield. The treatment of 5,7,3′,4′,5′-penta-*O*-benzyl-(−)-epigallocatechin with cesium hydroxide (CsOH), tetrabutyl ammonium iodide (TBAI), and various substituted benzyl bromide or various alkyl iodide reagents produced 5,7,3′,4′-tetra-*O*-benzyl-3-*O*-alkyl-(+)-epigallocatechins with 54–79% yields. The debenzylation of the 5,7,3′,4′-tetra-*O*-benzyl-3-*O*-alkyl-(+)-epigallocatechins was carried out with Pd/C in the

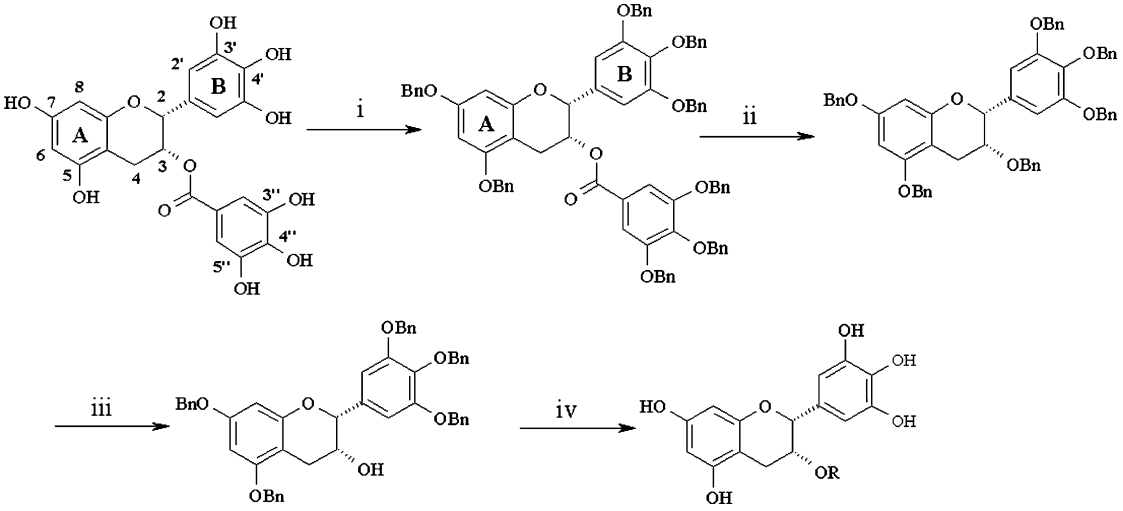


Fig. 1. Synthesis of 3-*O*-alkyl-(−)-epigallocatechins (1–7). Reagents and conditions: (i) BnBr, K_2CO_3, rt, 87%; (ii) NaOH, DMF/H_2O (4:1), 160 °C, 94%; (iii) CsOH, TBAI, R-X, rt, 54–79%; (iv) Pd/C, H_2, MeOH, rt, 74–82%.

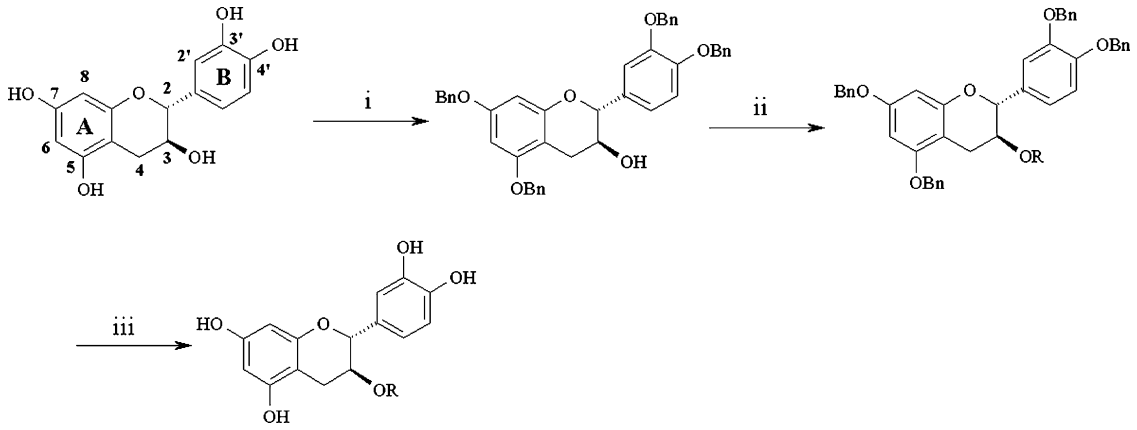


Fig. 2. Synthesis of 3-*O*-alkyl-(+)-catechins (8–16). Reagents and conditions: (i) BnBr, K_2CO_3, rt, 74%; (ii) CsOH, TBAI, R-X, rt, 46–72%; (iii) Pd/C, H_2, MeOH, rt, 78–89%.

presence of H_2 to produce compounds 1–7 with 74–82% yields.

2.2.2. Synthesis of 3-O-alkyl-(+)-catechins

The synthesis of the 3-*O*-alkyl-(+)-catechins is outlined in Fig. 2. For the introduction of the alkyloxyl group at the C-3 hydroxyl, the phenolic hydroxyl group of (+)-catechin (2 g, 6.89 mmol) was benzylated by treatment with benzyl bromide (4 eq.) and K_2CO_3 (6 eq.) to produce 5,7,3′,4′-tetra-*O*-benzyl-(+)-catechin with a 74% yield. The treatment of 5,7,3′,4′-tetra-*O*-benzyl-(+)-catechin with cesium hydroxide (CsOH), tetra-butyl ammonium iodide (TBAI), and various substituted benzyl bromide or various alkyl iodide reagents produced 5,7,3′,4′-tetra-*O*-benzyl-3-*O*-alkyl-(+)-catechins with 46–72% yields. The debenzylation of the 5,7,3′,4′-tetra-*O*-benzyl-3-*O*-alkyl-(+)-catechins was carried out with Pd/C in the presence of H_2 to produce compounds 8–16 with 78–89% yields.

2.3. Plaque reduction assay

Confluent monolayers of MDCK cells, cultured in a 6-well tissue culture plate (1×10^5 cells/cm^2), were infected with a mixture of approximately 300 PFU/mL of influenza A/Sydney/5/97 (H3N2) virus. After 45 min of virus adsorption, the solution was removed and the cells were washed twice with pre-warmed MEM, and replaced with overlay medium (DMEM containing 10 μg/mL trypsin, 1% low-melting agarose, no serum), containing catechins at different concentrations. After incubating cultures for 2–3 days at 37 °C in 5% CO_2, the cell monolayer was fixed with a 4% formaldehyde solution for 30 min. The agarose was removed by flowing water and plaques were stained with a 1% (w/v) crystal violet solution. The plaques were counted by visual examination and the degree of plaque inhibition was calculated relative to the control in the absence of catechins. The 50% effective concentration (EC_{50}) value was estimated by interpolation of the dose–response curve.

2.4. Virus yield reduction assay in egg allantoic fluid

The avian influenza A/Chicken/Korea/ms96/96 (H9N2) virus suspension containing 10-fold of egg infectious dose 50% (EID_{50})/50 μl was mixed with various concentrations of catechin derivatives (50 μl) for 1 h at room temperature. These solutions were inoculated into 10 fertilized eggs (11-day-old) via an allantoic route. Eggs were further incubated at 37 °C for 3 days. Allantoic fluids were harvested and titrated by hemagglutination (HA) assay. As controls, infected eggs with backbone compounds (EGC and C) and other antiviral compounds (EGCG, Amantadine, oseltamivir) or without test compounds were included throughout the experiment.

2.5. Hemagglutination inhibition assay

The hemagglutination inhibition (HI) assay was employed to evaluate the effects of the catechin derivatives on viral adsorption to target cells. Catechin solutions (25 μl) in serial two-fold dilutions in PBS were mixed with an equal volume of influenza virus solution (500 HAU/25 μl). After a 1 h incubation at room temperature, 50 μl of the solution was mixed with an equal volume of a 1% chicken erythrocyte suspension and incubated for 30 min at room temperature.

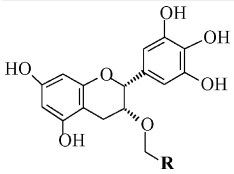
2.6. Cytotoxicity test by MTT assay

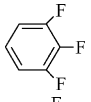
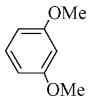
MDCK cells were grown (about 8000 cells/well; less than half confluency) in 96-well plates for 24 h. The media was replaced with media containing the serially diluted catechin derivatives and the cells were further incubated for 48 h. The culture medium was removed and 25 μl MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide] (Sigma) solution was added to each well and incubated at 37 °C for 4 h. After the removal of the supernatant, 50 μl DMSO was added to solubilize the formazan crystals and incubated for 30 min. The optical density was measured at 540 nm in an ELISA reader.

2.7. Statistical analysis

Experiments for the evaluation of both the EC_{50} and cytotoxicity concentration 50% (CC_{50}) values were carried out in triplicate and the Student's unpaired *t*-test was used to evaluate

Table 1
Anti-influenza (A/Sydney/5/97) virus activities and cytotoxicities of the 3-*O*-alkyl-(−)-epigallocatechin derivatives



Compound ID	*R*	EC_{50} (μM)[a]	CC_{50}[b] (μM)	SI[c]
1		36.9 ± 1.2	49.1 ± 2.7	1.3
2		51.1 ± 1.1	49.2 ± 1.9	0.9
3		61.1 ± 1.2	26.3 ± 3.1	0.4
4	$CH_2CH_2CH_3$	144 ± 1.5	159 ± 6.2	1.1
5	$CH_2(CH_2)_4CH_3$	32.1 ± 1.1	148 ± 3.1	4.6
6	$CH_2(CH_2)_7CH_3$	33.3 ± 1.4	178 ± 2.1	5.4
7	$CH_2(CH_2)_9CH_3$	28.1 ± 1.1	85.5 ± 1.1	3.0
EGC	–	>300	>1000	–

The values represent the mean ± S.E.M. for three independent experiments.
[a] EC_{50} represents the concentration of catechin derivatives necessary for a reduction in plaque number by 50% relative to control without test compound, and was calculated from the plaque reduction dose-response data.
[b] CC_{50} represents cellular toxicity to uninfected MDCK cells as determined by MTT assay.
[c] SI is the ratio of CC_{50} values to EC_{50} values.

the differences between the test samples and the control, PBS or catechins without chemical modification. A p value of <0.05 was considered statistically significant and a one-way ANOVA was used to evaluate the differences between the test samples. GraphPad PRISM software was used for statistical analysis.

3. Results

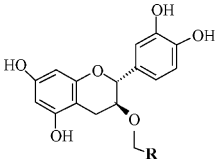
3.1. Evaluation of in vitro anti-influenza viral activity of catechin derivatives

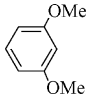
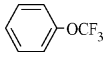
The antiviral activities of the (−)-epigallocatechin and (+)-catechin derivative compounds (Figs. 1 and 2) were initially evaluated by plaque reduction assay using MDCK cells. EGC and (+)-catechin, the primary catechin compounds, did not show any antiviral effects within the concentration range tested (300–1000 μM). As summarized in Tables 1 and 2, however, the 3-*O*-alkyl-(+)-catechin and 3-*O*-alkyl-(−)-epigallocatechin derivatives exhibited significantly higher antiviral activities when compared with EGC and (+)-catechin ($p < 0.05$). The EC_{50} values of these compounds for influenza A/Sydney/5/97 (H3N2) virus were within the range of 18.7–552 μM, depending on the nature of the 3-*O*-substitution. The most effective compound of the EGC-based derivatives was 3-*O*-dodecyl epigallocatechin (compound ID 7) with an EC_{50} of 28.1 μM. Compound ID 7 appeared, however, more toxic than 3-*O*-decyl epigallocatechin (compound ID 6) in MDCK cells (CC_{50} for compound ID 6 and 7 was 178 and 86 μM, respectively, and the selectivity index (SI) of 3-*O*-dodecyl epigallocatechin was approximately twice that of 3-*O*-dodecyl epigallocatechin. Of the (+)-catechin-based derivatives, 3-*O*-decyl catechin (compound ID 15) appeared to be the best compound in regard to both the EC_{50} (18.7 μM; lowest of all compounds tested) and the SI (4.5-fold; highest of all (+)-catechin based compounds tested). Our analysis showed that the inactivation kinetics of influenza virus by catechins is extremely fast, usually lowering the viral titer by 2 logs within 10–30 min of direct contact with the virus (data not shown).

3.2. Structure-antiviral activity relationships

Among the 3-*O*-alkyl-epigallocatechin derivatives (ID 1–7), the EC_{50} values of compounds ID 1–3 having a substituted aromatic group were 36.9–61.1 μM. The EC_{50} values of compounds ID 5–7 that contain aliphatic chains of varying lengths were 28.1–32.1 μM. Therefore, aliphatic chains contributed more to the antiviral activity than did the aromatic group by 1.5–2.0-fold. Considering the cytotoxic effects, as reflected by the SI, the antiviral efficacy is further increased up to five-fold. Increasing carbon chain length from C3 to C6 enhanced the antiviral activity by 17-fold (compare compounds 4 and 5), whereas a further increase in chain length to C9 or C11 did not contribute significantly to antiviral activity. A similar correlation between antiviral activity and the nature of the 3-position substitution was also observed with the (+)-catechin derivatives. Compounds ID 13–16 that had aliphatic substitutions exhibited greater inhibitory effects on plaque formation than did compounds ID 8–12 that had aromatic substitutions. Antiviral activity was enhanced by increased chain lengths, although this effect was less pronounced in the EGC derivatives. Our results

Table 2
Anti-influenza (A/Sydney/5/97) virus activities and cytotoxicities of the 3-*O*-alkyl-(+)-catechin derivatives



Compound ID	R	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
8		38.6 ± 1.2	35.0 ± 1.9	0.9
9		30.2 ± 1.1	45.1 ± 2.0	1.5
10		91.4 ± 5.0	114 ± 1.7	1.3
11		26.9 ± 1.1	32.3 ± 1.6	1.2
12		53.2 ± 1.2	97.0 ± 3.3	1.8
13	$CH_2CH_2CH_3$	72.3 ± 1.2	186 ± 3.4	2.6
14	$CH_2(CH_2)_4CH_3$	25.6 ± 1.1	54.3 ± 1.2	2.1
15	$CH_2(CH_2)_7CH_3$	18.7 ± 1.1	84.3 ± 1.1	4.5
16	$CH_2(CH_2)_9CH_3$	31.2 ± 1.2	66.5 ± 1.8	2.1
(+)-C	–	>600	>600	–

The values represent the mean ± S.E.M. for three independent experiments.
[a] EC_{50} represents the concentration of catechin derivatives necessary for a reduction in plaque number by 50% relative to control without test compound, and was calculated from plaque reduction dose–response data.
[b] CC_{50} represents cellular toxicity to uninfected MDCK cells as determined by MTT assay.
[c] SI is the ratio of CC_{50} values to EC_{50} values.

also suggest that the 3-position substitution in the galloyl moiety is much more important than of the 5′-OH group in the trihydroxyphenyl moiety in the primary catechin structure. For example, compounds ID 1 and 8 that have the same 3,4,5-trifluorobenzyl group exhibited similar ($p > 0.7$) EC_{50} values, 36.9 and 38.6 μM, respectively. A similar observation was made for compounds ID 7 and 16; both carry the same substitution (i.e., a dodecyl group) and exhibited similar ($p > 0.9$) EC_{50} values, 28.1 and 31.2 μM, respectively. Overall, the substitution at the 3-position was much more important than the 5′-OH group in the trihydroxyphenyl backbone for anti-influenza viral activity.

3.3. Antiviral effects on avian influenza virus in embryonated eggs

Compounds ID 6, 7, 15, and 16, that exhibited inhibitory effects in an in vitro plaque reduction assay, were further examined in fertilized embryonated eggs as an appropriate substrate for the avian influenza viruses. Various concentrations (200–1.25 μM) of each compound were mixed with an equal volume of influenza A/Chicken/Korea/ms96/96 (H9N2) virus (10 EID_{50}), and inoculated in embryonated eggs. Eggs were incubated for 3 days at 37 °C and allantoic fluids were harvested. The viral yield was estimated by hemagglutination assay. Oseltamivir, amantadine, (+)-C, EGC, EGCG and none-treated sample were included as controls. The avian virus used in this experiment was a low pathogenic virus; because of low embryonic lethality, a virus titre of greater than or equal to 10^7 EID_{50}/mL could be attained in the absence of antiviral compounds. As shown in Table 3, a pronounced inhibition in virus propagation was observed at concentration of 5.1 μM for compounds ID 15 and 16 and 9.6 μM and 10.1 μM for compounds ID 6 and 7, respectively. Under the same experimental conditions, the growth of avian viruses was not significantly affected

Table 3
Antiviral effects on avian influenza virus in embryonated eggs

Viruses	Compound ID MIC[a] (μM)								
	6	7	15	16	EGC	(+)-C	EGCG	Osel-tamivir	Aman-tadine
A/Chicken/Korea/ ms96/96 (H9N2)	9.6 ± 2.1	10.1 ± 1.5	5.1 ± 1.7	5.1 ± 1.7	>1000	>1000	>1000	250–500	>1000

The values represent the mean ± S.E.M. for three independent experiments.
[a] MIC represents the minimum inhibitory concentration of catechin derivatives at which complete inhibition of virus in embryonated eggs was observed.

Table 4
Inhibitory effects of catechin derivatives on virus adsorption to chicken red blood cells

Viruses	Compound ID MIC[a] (μM)				
	6	7	15	16	Controls[b]
A/Sydney/5/97(H3N2)	20.8 ± 7.2	41.7 ± 14.4	83.3 ± 28.9	41.7 ± 14.4	>800
A/X-31(H3N2)	20.8 ± 7.2	33.3 ± 14.4	66.7 ± 28.9	33.3 ± 14.4	>800
A/PR/8(H1N1)	25.0 ± 0.0	25.0 ± 0.0	50.0 ± 0.0	20.8 ± 7.2	>800
A/Japan/305/57(H2N2)	41.7 ± 14.4	116.7 ± 76.4	50.0 ± 0.0	33.3 ± 0.0	>800
A/Chicken/Korea/ms96/96 (H9N2)	35.4 ± 13.0	54.2 ± 7.2	35.4 ± 13.0	21.9 ± 5.4	>800
B/Lee/40	25.0 ± 0.0	33.3 ± 14.4	66.7 ± 28.9	50.0 ± 43.3	>800

The values represent the mean ± S.E.M. for three independent experiments.
[a] MIC represents the concentration of catechin derivatives necessary to completely inhibit adsorption of virus onto chicken red blood cells by hemagglutination inhibition assay.
[b] Controls are EGC, (+)-C, amantadine and oseltamivir.

by EGCG that previously exhibited strongest anti-influenza viral activity among natural green tea catechins (Song et al., 2005). Another well known anti-influenza virus drugs, amantadine, an M2 ion channel blocker, failed to show significant effect even at 1 mM. Oseltamivir, a specific neuraminidase inhibitor, exhibited an inhibitory effect at a much high concentration (250–500 μM (MIC)) than the catechin derivatives. The data suggest that, in contrast to oseltamivir that functions at the virus-release step at a later stage of infection, the catechin derivatives exerted a prolonged inhibitory effect possibly due to increased stability in ovo.

3.4. Inhibitory effects of compounds on virus adsorption onto chicken red blood cells (RBCs)

Influenza A and B viruses, including avian influenza virus, have the ability to adsorb onto chicken RBCs, resulting in hemagglutination. We therefore estimated the minimum concentration of catechin derivatives that interferes with viral adsorption to RBCs, resulting in hemagglutination inhibition (HI). As shown in Table 4, all of the tested compounds (ID 6, 7, 15, and 16) exhibited complete inhibition of viral adsorption onto RBCs in a concentration range of 20–120 μM, depending on the virus type tested. Overall, compound ID 6 was the most effective among all derivatives, with an MIC less than 50 μM for both human and avian influenza viruses. Remarkably, HI was observed for all virus subtypes, including H1N1, H2N2, H3N2 human influenza A virus, human influenza B virus, and H9N2 avian influenza virus. Compounds ID 7, 15, and 16 completely inhibited viral adsorption onto RBCs at a concentration range of 20.8–116.7 μM. Influenza A/Japan/305/57 (H2N2) virus was relatively insensitive to compound ID 7 (MIC ≈120 μM), although the reason for the observed discrepancy was not explored further. Amantadine and oseltamivir did not show any inhibitory effects on RBC adsorption even at the highest concentration tested (2 mM), as expected from their different inhibitory modes of action. In parallel, EGC and (+)-catechin, without any 3-position substitution failed to show an inhibitory effect (Table 4 and controls). We conclude from these data that the 3-position substitution crucially affected the antiviral activity against influenza viruses and that inhibition is mainly mediated by an interaction with hemagglutinin on the viral membrane.

4. Discussion

Two classes of antiviral drugs are currently in use for both prophylactic and therapeutic treatments of influenza viruses. Amantadine and rimantadine, which block the ion channel activity of the M2 protein (Hay et al., 1985; Pinto et al., 1992), are not very effective due to the frequent emergence of drug-resistant viral variants and the lack of activity against the influenza B virus (Betakova et al., 1996; Stiver, 2003). The other class comprises the NA inhibitors, zanamivir and oseltamivir, which act in the later stages of established infection by inhibiting the release of virions from infected cells (Kim et al., 1997; von Itzstein et al., 1993). The effectiveness of these compounds is also compromised due to the emergence of drug-resistant viral variants that escape interaction with the active site of neuraminidase (Gubareva et al., 2001; Le et al., 2005; Thompson et al., 2004). Therefore, there has been a constant need for broad-spectrum antiviral drugs that exert inhibitory effects against currently circulating human viruses as well as newly emerging avian influenza viruses. We have previously described the anti-influenza viral activity of green tea catechin, EGCG, and ECG; the structure-function relationships of these catechin compounds suggested that the 3-galloyl group of the catechin skeleton plays an important role in its antiviral activity (Song et al., 2005). Consistent with our previous results, substitution group at position 3, subjected to its nature and size, serves as the main structural principle for the antiviral activity among catechin derivatives. Here we substituted the galloyl group of epigallocatechin and (+)-catechin with aromatic groups and alkyl chains of various lengths. Depending on the nature of the substitution, their antiviral activities were greatly enhanced ($p < 0.0001$) compared with the backbone compounds.

Overall, aliphatic chains were more effective than were aromatic substitutions, and most prominently, compounds 6 and 15 of moderate chain length (C9) exhibited the strongest antiviral activities while maintaining high SI values. The increases in antiviral activities of compounds with aromatic substitutions were predominantly compromised by similar increases in cytotoxicities rendering SI value less meaningful. The lengths of the aliphatic chains should be greater than three to be effective in increasing antiviral activity. These observations are consistent with previous reports. For example, the antibacterial activity

of 3-*O*-acyl-catechins correlated with chain length at the 3-hydroxyl position, exhibiting maximal activity with a 8–10 carbon chain (Stapleton et al., 2004). Likewise, EGC with an acyl chain of 8–11 carbon atoms showed marked inhibition of Epstein–Barr virus activation (Uesato et al., 2003).

How could the enhancement of anti-influenza activity by an aliphatic side chain be explained? One possibility is that the hydrophobic aliphatic chain interacts with the influenza viral envelope, causing a change in the structural integrity of the viral membrane. Whether the physical changes in viral membrane also impacts the integrity of the hemagglutinin trimer embedded in the viral membrane remains to be investigated. Considering the crucial role of the HA protein for the binding of the cellular receptor sialic acid during the initial phase of infection (Weis et al., 1988), any changes in physical integrity or conformation mediated by catechin molecules is expected to importantly inhibit influenza infection. This explanation is in agreement with the apparent interference of the catechin derivatives on the attachment of viruses to RBCs that is mediated by the sialic acid receptor–HA interaction. Moreover, if we pretreated the catechin derivatives on MDCK cells before the virus infection, we could not observe any plaque reduction effects (data not shown). This also support that the catechin derivatives act on virus particle directly although it is still possible that the compounds may also affect other steps in influenza virus life cycle (Song et al., 2005).

The antiviral activities of the catechin derivatives were most prominent in fertilized eggs and distinctively higher than previously evaluated catechins, such as EGCG (Table 3). Presumably, this difference was associated with the stability of compounds especially in physiological condition. To avoid potential attack by esterase and other factors that may negatively influence the stability, we replaced the ester bond with ether at the 3-position which is significantly associated with the antiviral activity. It is possible that the modification may increase the half life of the compounds in allantoic fluids. In addition, there was about two-fold difference in antiviral activities between two groups of catechin derivatives, EGC backbone (Compound ID 6 and 7) and (+)-catechin (Compound ID 15 and 16). This result is associated with the hydroxyl group in 5′ position of the B ring. Whether deprotonation of the hydroxyl group is related with the stability remains to be explored. Our own analysis showed that the half life of catechin derivative was about two-fold higher than EGCG in pH 7.4 solution, and HPLC analysis showed that, after 10 h incubation with this solution, the concentration of catechin derivatives were five- to six-fold higher than EGCG (unpublished results).

The antiviral activities of the catechin derivatives are distinctively different from previously developed inhibitors, such as amantadine or oseltamivir. Oseltamivir specifically interacts with the NA protein, inhibiting cleavage of the sialic acid moiety and the release of newly budded virions from infected cells. Tighter binding to the NA target leads to enhanced efficacy as exemplified by peramivir (Bantia et al., 2006), suggesting that this interaction is essentially reversible, as is the case with amantadine which interacts with the M2 proton channel on the viral envelope (Drusano et al., 2001). It is likely that the initially bound oseltamivir dissociates from NA, especially in the allantoic cavity, by competition with biomolecules in the allantoic fluid. A relatively large volume (about 10 mL) of allantoic fluid in the fertilized eggs would also shift the equilibrium in favor of inhibitor dissociation. In contrast, the interaction of the catechin derivatives with the HA molecule in the viral membrane may be irreversible and results in a virucidal effect (Tables 1 and 2). We think that is the reason why the catechin derivatives are more effective than oseltamivir in fertilized eggs although oseltamivir has lower EC_{50} value, 0.1–1 μM, than catechin derivatives in plaque reduction assay (data not shown).

It should be noted that a benzamide class hemagglutinin-specific inhibitor has been previously identified that has antiviral activity against H1 and H2 but not for H3 and B viruses (Luo et al., 1996, 1997). In contrast, the catechin derivatives described in this report exhibited a broad spectrum of activity against all influenza virus subtypes tested, including H1, H2, H3, H9, and influenza B virus (Table 4). The results further extend previous studies on natural catechin (Song et al., 2005). The antiviral activity could also be extended to HPAI (highly pathogenic influenza virus) clades including H5N1, and may have implication in minimizing animal to human transmission of H5N1 viruses (Song and Seong, 2007). We suggest that the catechin derivatives interfere with the HA-viral membrane interactions and alter the physical properties of the HA protein, rendering it less fusogenic, distinct from the action of RC2, a θ-defensin retrocyclin2 (Leikina et al., 2005). Whether the aliphatic side chain, resembling the lipid moiety of pathogenic bacterial cell wall, would help stimulate an innate immune system thus further enhancing the therapeutic effect merits further investigation.

In conclusion, catechin derivative compounds that contain carbon chains of moderate lengths have potent anti-influenza viral activities both in vitro and in ovo. These compounds are likely to inhibit the initial stage of influenza virus infection, rendering HA less fusogenic. A detailed action mechanism and the clinical relevance for controlling the currently circulating human influenza and emerging avian influenza viruses remain to be explored.

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