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In vitro anti-influenza virus and anti-inflammatory activities of theaflavin derivatives

Mian Zu [a], Fan Yang [a], Weiling Zhou [a], Ailin Liu [a,b,*], Guanhua Du [a,b,*], Lishu Zheng [c,*]

[a] Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China
[b] Beijing Key Laboratory of Drug Target Research and Drug Screening, Beijing 100050, PR China
[c] Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China

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ABSTRACT

The theaflavins fraction (TF80%, with a purity of 80%) and three theaflavin (TF) derivatives from black tea have been found to exhibit potent inhibitory effects against influenza virus *in vitro*. They were evaluated with a neuraminidase (NA) activity assay, a hemagglutination (HA) inhibition assay, a real-time quantitative PCR (qPCR) assay for gene expression of hemagglutinin (HA) and a cytopathic effect (CPE) reduction assay. The experimental results showed that they all exerted significant inhibitory effects on the NA of three different subtypes of influenza virus strains [A/PR/8/34(H1N1), A/Sydney/5/97(H3N2) and B/Jiangsu/10/2003] with 50% inhibitory concentration (IC_{50}) values ranging from 9.27 to 36.55 μg/mL, and they also displayed an inhibitory effect on HA; these inhibitory effects might constitute two major mechanisms of their antiviral activity. Time-of-addition studies demonstrated that TF derivatives might have a direct effect on viral particle infectivity, which was consistent with the inhibitory effect on HA. Subsequently, the inhibitory effect of TF derivatives on the replication of the viral HA gene as assayed by qPCR and on the nuclear localization of the influenza virus vRNP further demonstrated that they may primarily act during the early stage of infection. Interestingly, besides the activity against functional viral proteins, TF derivatives also decreased the expression level of the inflammatory cytokine IL-6 during viral infection, expression of which may result in serious tissue injury and apoptosis. Our results indicated that TF derivatives are potential compounds with anti-influenza viral replication and anti-inflammatory properties. These findings will provide important information for new drug design and development for the treatment of influenza virus infection.

1. Introduction

Influenza viruses cause worldwide outbreaks and pandemics in humans and animals every year with high morbidity and mortality (Miller et al., 2009). The severe 'Spanish flu' pandemic between 1918 and 1919 resulted in hundreds of millions of cases and led to approximately 20–50 million human deaths (Taubenberger et al., 2001). The enveloped, negative-stranded influenza virus belongs to the family *Orthomyxoviridae*. It is divided into three types, A, B and C, based on the antigenic and genetic differences of the inner proteins and genome structure. The viral genome of the influenza A virus contains eight segmented ssRNAs, which encode 11 viral proteins and peptides. The two viral glycoproteins located on the surface of the virus, hemagglutinin (HA) and neuraminidase (NA) are crucial for the replication and infectivity of the influenza virus (Fiers et al., 2004).

Currently, two classes of drugs have been approved by the FDA for influenza virus prevention and therapy, M2 ion-channel inhibitors (*i.e.*, amantadine and rimantadine) and neuraminidase inhibitors (*i.e.*, oseltamivir and zanamivir). The former is effective only against type A viruses and drug resistance has become widespread (Pielak and Chou, 2010), while the later, which is effective against both type A and B viruses, is also facing drug-resistance in new strains (Dolin, 2011; Longtin et al., 2011; Hurt et al., 2011; Ramirez-Gonzalez et al., 2011). These reasons urged us to investigate new antiviral drugs against influenza virus.

Plants have a long history in the treatment of infectious diseases caused by viruses in traditional Chinese medical practices. Numerous experiments have shown that polyphenolic compounds exert a wide range of pharmacological activities, including antinociceptive, antioxidative, antibacterial, anticancer and antiviral (Hajhashemi et al., 2011; Yook et al., 2010; Szliszka et al., 2011). Polyphenolic catechins from green tea were studied for their ability to inhibit influenza virus replication in cell culture

* Corresponding authors. Address: Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China. Tel.: +86 10 83150885; fax: +86 10 63017757 (A. Liu), tel.: +86 10 63165184; fax: +86 10 63017757 (G. Du), tel.: +86 10 83549762; fax: +86 10 63544432 (L. Zheng).
E-mail addresses: liuailin@imm.ac.cn (A. Liu), dugh@imm.ac.cn (G. Du), zhenglishu2000@sina.com (L. Zheng).

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(Song et al., 2005). Especially, as one of the major catechins of green tea polyphenols, (−)-Epigallocatechin gallate (EGCG) inhibits influenza A virus endonuclease with chemical groups of catechin as an important determinant for this activity (Kuzuhara et al., 2009). Theaflavins (TFs), a group of polyphenolic compounds, are a major component of black tea, which are made by the oxidation and dimerization during the manufacture of black tea and oolong tea from green tea catechins and account for 2–6% of the dry weight of solids (Robert, 1958). Up to now, more than 28 TF derivatives have been isolated, and the most abundant TFs in black tea are theaflavin (TF), theaflavin-3-gallate (TF-3-G), theaflavin-3′-G (TF-3′-G), theaflavin-3,3′-DG (TF-3,3′-DG). The bioavailability and bioactivity of tea polyphenols (PP) and theaflavins in mouse tissue indicated that PP and theaflavins were found in the small and large intestine, liver, and prostate in conjugated and free forms. Although tea PP were not detectable in serum for the human study, the inhibitory effect against ex vivo LNCaP prostate cancer cell proliferation was obtained when treated the cells with media containing patient serum collected after black tea and green tea consumption (Henning et al., 2006). However, antiviral activity in theaflavins has not been reported before. To discover new drugs against influenza virus, we have established a NA inhibitory assay and carried out high throughput screening (HTS) (Liu et al., 2008a,b). The HTS results showed that theaflavin extracts and theaflavin derivatives including TF-3-G, TF-3′-G and TF-3,3′-DG exhibited NA inhibition; therefore, their anti-influenza virus effect and the underlying mechanism were further investigated in the present study.

2. Materials and methods

2.1. Cells and virus strains

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). A549 cells (an epithelial cell line through explant culture of lung carcinomatous tissue) were maintained in RPMI1640 medium containing 10% FBS. Before infection, cells were washed with PBS (pH 7.2–7.4) and cultured in media supplemented with 2 μg/mL trypsin TPCK treated from bovine pancreas (Sigma–Aldrich, Lot# 031M7358V).

The human influenza virus strains A/PR/8/34(A/H1N1), A/Sydney/5/97(A/H3N2) and B/Jiangsu/10/2003 were kindly donated by the Institute for Viral Disease Control and Prevention, China Centers for Disease Control and Prevention. Viral stocks of these laboratory-adapted strains were prepared by passaging them in 9-day-old embryonated chicken eggs for 48 or 72 h.

2.2. Extract and compounds

The 80%TF containing of TF, TF-3-G, TF-3′-G and TF-3,3′-DG with more than 80% purity was provided by DeHe Bio-technology Company in Jiangsu province in China. The three TF derivatives, specifically TF-3-G, TF-3′-G and TF-3,3′-DG, were provided by the National Center for Pharmaceutical Screening in the Institute of Materia Medica, Chinese Academy of Medical Sciences, China (Fig. 1), and their purity was greater than 98%. Ribavirin with ⩾98% purity (Sigma–Aldrich, Lot# 020M4003) and the active form of Tamiflu, oseltamivir carboxylate/oseltamivir acid (J&K Scientific Ltd.) were used as reference compounds in CPE and NA inhibition assay, respectively. All the compounds and extracts were dissolved in DMSO to 10 mg/mL as a stock solution. Serum-free medium or 2-(*N*-morpholino) ethanesulfonic acid (MES) hydrate buffer or saline was used as the dilution buffer in the follow-up experiments.

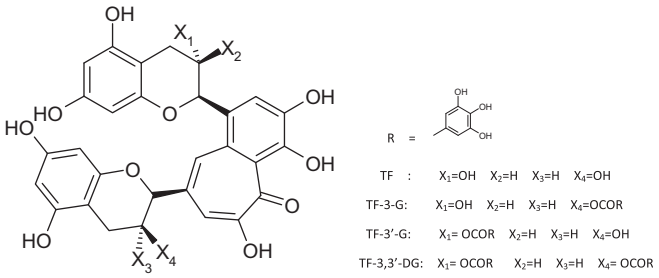

Fig. 1. Chemical structures of theaflavin and its derivatives.

2.3. Neuraminidase inhibition assay

A neuraminidase (NA) inhibition assay was developed to detect antiviral effects and to determine the potential mechanism of "hit" compounds. The NA inhibition assay was conducted using NAs from three types of influenza viruses as follows: A/PR/8/34(H1N1), A/Sydney/5/97(H3N2) and B/Jiangsu/10/2003. Influenza virus NA activity was assayed by quantifying the fluorescent product resulting from the cleavage of the substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate solution (MUNANA) by NA (Potier et al., 1979). The reaction mixture consisted of the tested compounds, virus (as the source of NAs) and MUNANA in 32.5 mM MES buffer (containing 4 mM $CaCl_2$, pH = 6.5) in a 96-well plate. After incubation for 60 min at 37 °C, the reaction was terminated with 150 μL 34 mM NaOH and the fluorescence of the mixture was recorded for the excitation wavelength 360 nm and emission wavelength 450 nm (Kwon et al., 2010). The inhibition ratio was obtained using the equation:

$$\text{Inhibition activity } (\%) = (F_{\text{Virus}} - F_{\text{Sample}})/(F_{\text{Virus}} - F_{\text{Substrate}}) * 100$$

where F_{Virus} is the fluorescence of the influenza virus control (virus, buffer and substrate), $F_{\text{Substrate}}$ is the fluorescence of the substrate control (buffer and substrate), and F_{Sample} is the fluorescence of the tested samples (virus, sample solution and substrate). Subsequently, the 50% inhibitory concentration (IC_{50}) was determined by extrapolation of the results from various doses tested using a linear equation. The experiment was repeated at least twice with a similar finding each time. At least three independent measurements were collected to determine the mean and SD values.

2.4. Cytotoxicity test

MDCK cells (2×10^4 per well) or A549 cells (1×10^4 per well) grown in 96-well plate for 24 h were washed once with serum-free medium and were then treated with TF derivatives at the concentrations between 1 and 200 μg/mL or mock control solutions (DMSO of 2%) at 37 °C and 5% CO_2 for 72 h. Proliferation of cells was measured by the MTS assay. According to the Manufacturer's protocol, 2 mg/mL MTS (Promega) completely dissolved in Dulbecco's phosphate-buffered saline (DPBS) combined with 0.92 mg/mL PMS (Sigma) was added to the cell culture medium for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance was recorded at 490 nm using an ELISA plate reader. Cytotoxicity of the compounds was estimated by comparison of the cell survival rate of TF derivatives treated cells with that of mock-treated. The survival rate of mock-treated control was set as 100%.

2.5. Cytopathic effect (CPE) reduction assay

MDCK cells were seeded into 96-well plates at 2×10^4 cells per well 24 h prior to infection and incubated at 37 °C 5% CO_2. To evaluate the pharmacological characteristics of TF derivatives,

three different time points for drug administration were utilized in our experiments. (1) Pre-incubation treatment assay: A/PR/8/34(H1N1) at MOI of 0.1 was pre-incubated with serial dilutions of test samples for 1 h at 4 °C before adding virus combined with samples to MDCK cells for virus titer determination. (2) Simultaneous treatment assay: serial dilutions of the test samples were added to the cells at the same time as influenza virus strain (MOI 0.1). (3) Post-treatment assay: the test samples were added to the cells 1 h after the adsorption of the influenza virus A/PR/8/34 (MOI 0.1). The positive control was ribavirin. At 24 h post-infection, microscopy was performed to determine the antiviral effect, which was expressed as the concentration that reduced the virus-induced CPE by 50% (50% effective concentration, [EC_{50}]) (Liu et al., 2008a,b), and to determine the nonspecific cytotoxicity of the theaflavins, expressed as the maximal non-cytotoxic concentration (MNCC). The data were confirmed using a crystal violet-based cell viability assay (Schmidtke et al., 2001), and the resulting spectrophotometric data were used to calculate the EC_{50} and the 50% cytotoxic concentration (CC_{50}) (Liu et al., 2008a,b). The experiment was repeated at least three times. At least nine parallel measurements were obtained for the mean and SD values.

2.6. Hemagglutination inhibition (HI) assay

The HI assay was performed to evaluate the inhibitory effects of TF derivatives on viral adsorption into target cells. The hemagglutinin (HA) titers of viral stocks were initially determined by the standard HA assay in round-bottomed 96-well microplates. HI tests were subsequently performed using 4 times the HA units (HAU) of virus per well. A volume of 25 μL of the theafavins and TF derivatives from a twofold serial dilution in saline were added into the microwell plate prior to the addition of an equal volume of virus (4HAU). Subsequently, 50 μL of guinea pig erythrocytes (1% v/v in saline) were added to each well. The hemagglutination reaction results were read after incubation for 1 h at room temperature (Chen et al., 2010; Kwon et al., 2010). The experiment was repeated twice independently with similar results, with three parallel measurements each time.

2.7. Quantitative real-time PCR assay

A549 cells were grown to approximately 90% confluence in 6-well plates with 4×10^5 cells per well at 37 °C 5% CO_2, infected with influenza virus at MOI 0.1 and cultured in the presence of TF derivatives at concentrations of 3 or 10 μg/mL. Cells were lysed by directly adding 500 μL TRizol (Invitrogen) to each well following medium removal 7 h after A/PR/8/34(H1N1) virus infection, and the cell lysates were collected (Kwon et al., 2010). To determine the expression level of mRNA from the HA gene of the influenza virus, a total RNA isolation procedure was carried out in accordance with the instructions. Total RNA was reverse transcribed into cDNA using the *TransScript* First-Strand cDNA Synthesis SuperMix (TransGen Biotech) according to the Manufacturer's protocol. The enzyme was inactivated at 85 °C for 5 min. The cDNA was stored at −20 °C or directly used for qPCR. The primer sequences, which were designed by Primer-BLAST from NCBI, for qPCR of the influenza virus HA gene were 5′-CCTGCTCGAAGACAGCCACAACG-3′ (sense) and 5′-TTCCCAAGAGCCATCCGGCGA-3′ (antisense). GAPDH primers were used as internal control of cellular RNAs: 5′-AGGCGTCGGAGGGCCCCCTC-3′ (sense) and 5′-AGGGCAATGCCAGCCCCAGCG-3′ (antisense). qPCR was conducted using cDNA, the sense and antisense primers, and SsoFast EvaGreen PCR 2× master mix (Bio-Rad). Cycling conditions for the qPCR were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s, 65 °C for 30 s and 72 °C for 1 s. Finally, a melting curve was generated by increasing the temperature at a gradient of 0.05 °C from 65 to 95 °C. qPCR was conducted using the CFX96 Realtime PCR system (Bio-Rad). The data were analyzed with the Bio-Rad CFX manager using the mode for normalized expression ($2^{-\Delta\Delta Cq}$). The experiment was performed for three times with three parallel measurements each time.

2.8. Indirect immunofluorescence microscopy

Confluent monolayers A549 cell infected with influenza virus A/PR/8/34 (H1N1) at MOI 0.1 were treated with the three TF derivatives in a dose range of 3–30 μg/mL. At 12 h post-infection, the supernatant was removed, and the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.3% Triton-X100 for 10 min at room temperature and blocked with 5% BSA (Amoresco) for 2 h at 4 °C. After labeling with a mouse monoclonal antibody directed against influenza A virus nucleoprotein (NP) [9G8] (abcam, ab43821) (1:100 in 5% BSA) at 4 °C overnight and 5% Anti-Mouse IgG (Alexa Fluor® 488 Conjugate) as a secondary antibody (Cell Signaling Technology. #4408) (1:50 in 5% BSA) at 4 °C overnight, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL in methanol) at 37 °C for 10 min. Fluorescence was observed using an Olympus fluorescence microscope (Olympus, Germany) (Geiler et al., 2010). In total, three independent experiments were performed with similar results, in which at least three photographs were randomly taken for each well.

2.9. Cytokine secretion assay

Confluent monolayers A549 cells in 96-well plate at 37 °C 5% CO_2 infected with influenza virus (H1N1) at MOI 0.1 were treated with TF derivatives at the serially diluted, non-cytotoxic concentrations of 1, 5 or 25 μg/mL. Supernatants from mock or H1N1-infected cells at 24 h post-infection were compared for the expression of IL-6 by ELISA (4A biotech Co. Ltd.) according to the Manufacturer's protocol (Geiler et al., 2010). The experiment was repeated twice with similar results in three parallel measurements.

2.10. Statistical analysis

All data are given as the mean ± SD. Statistical analysis of the results was performed with Students' *t*-test. p values of <0.01 were considered to be statistically significant.

3. Results

3.1. Neuraminidase inhibition activity

To detect any antiviral effect of TF derivatives and to determine the corresponding mechanisms, an NA activity assay was performed. The results (Table 1) showed that TF derivatives all exhibited an inhibitory effect on the NAs from three subtypes of influenza virus strains with IC_{50} values ranging from 10.67 ± 0.31 to 49.60 ± 4.74 μM. The inhibitory effects of the three TFs against influenza A virus were more potent than influenza B virus, and the inhibitory effect against the H3N2 subtype virus was stronger than against H1N1. Interestingly, the positive control, oseltamivir carboxylate, showed the same inhibitory trend against the three types of NAs. TF-3,3′-DG displayed the highest inhibitory activity against the NAs, with an IC_{50} of 10.67 ± 0.31 μM.

3.2. Antiviral activities in a CPE reduction assay against influenza A virus strains

To confirm the antiviral activity of TF derivatives, their reduction of influenza virus-induced cytopathic effects were evaluated.

Table 1
IC_{50} values of NA inhibitory activity of TF derivatives and Oseltamivir carboxylate.

Samples	IC_{50}		
	A/PR/8/34(H1N1)	A/Sydney/5/97(H3N2)	B/Jiangsu/10/2003
TF80% (μg/mL)	11.65 ± 0.66	25.72 ± 0.81	27.98 ± 3.17
TF-3-G (μM)	31.91 ± 4.18	13.29 ± 2.32	49.60 ± 4.74
TF-3′-G (μM)	35.23 ± 7.75	18.26 ± 1.07	49.23 ± 4.20
TF-3,3′-DG (μM)	26.25 ± 6.20	10.67 ± 0.31	42.07 ± 2.16
Oseltamivir carboxylate (nM)	15.57 ± 1.73	8.88 ± 2.17	31.60 ± 2.88

Table 2
CC_{50} values of TF derivatives in MDCK and A549 cells.

Samples	CC_{50} (μg/mL)	
	A549	MDCK
TF80%	73.5	174.3
TF-3-G	172.9	177.1
TF-3′-G	164.2	167.8
TF-3,3′-DG	76.7	143.4

First, a cell proliferation and viability assay based on MTS was performed to determine the nonspecific cytotoxicity of the TF compounds for MDCK cells. The CC_{50} of TF80%, TF-3-G, or TF-3′-G, or TF-3,3′-DG was 174.3, 177.1, 167.8, 143.4 μg/mL, respectively. For A549 cells, the CC_{50} values were 73.5–172.9 μg/mL (Table 2). No significant cellular toxic effect was observed within the concentration range of 1–30 μg/mL of TF derivatives in both MDCK and A549 cells when the compounds and cells were incubated together at 37 °C for 24 h in a humidified atmosphere of 5% CO_2. The data from the CPE reduction assay demonstrated that TF derivatives all exhibited inhibitory antiviral effects, not only in the pre-incubation treatment assay and post-treatment assay after viral adsorption, but also in a simultaneous treatment assay when co-incubated to virus and TF compounds. When the virus was pre-incubated with test samples, TF-3,3′-DG afforded the most active inhibitory effect, with the IC_{50} of 0.70 ± 0.13 μg/mL. TF-3-G showed the lowest inhibitory effect, with an IC_{50} of 6.89 ± 2.70 μg/mL. TF80% and TF-3′-G had an intermediate effect, with an IC_{50}s of 3.79 ± 0.58 and 1.74 ± 0.31 μg/mL, respectively (Fig. 2). As positive controls, ribavirin and oseltamivir carboxylate showed an inhibitory effect against A/PR/8/34(H1N1) in pre-incubation treatment, with the IC_{50} values of 5.04 and 1.85 μg/mL, respectively. The IC_{50} values of TF derivatives ranged from 12.45 ± 1.07 to 20.93 ± 3.47 μg/mL in the post-treatment assay in which TF-3′-G showed the strongest suppressive activity. The IC_{50} values ranged from 7.23 ± 3.16 to 12.38 ± 3.57 μg/mL for simultaneous treatment, and TF-3,3′-DG showed the most potent inhibitory effect. Interestingly, both TF derivatives and ribavirin showed efficacy against CPE in three kinds of administrations: Pre-incubation treatment > Simultaneous treatment > Post-treatment (Fig. 2).

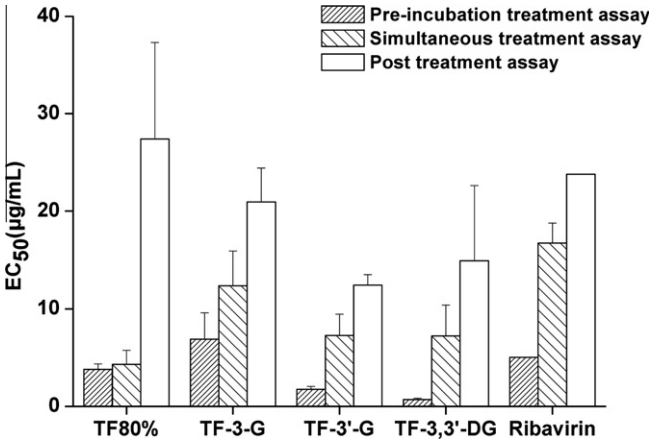

Fig. 2. *In vitro* time-of-addition studies on anti-influenza viral activity of the TF derivatives by CPE reduction assay in MDCK cells. The cells were divided into three groups. Group 1 (Pre-incubation treatment): serial dilutions of TF derivatives from 0.1 to 30 μg/mL were pre-incubated with influenza virus (A/PR/8/34, H1N1, MOI 0.1) for 1 h at 4 °C before added to the cells. Group 2 (Simultaneous treatment): Virus A/PR/8/34 (MOI 0.1) together with serial dilutions of the TF derivatives were added to cell monolayers cultivated in 96-well culture plates. Group 3 (Post-treatment): Samples were added to cell monolayers after influenza virus adsorption at 33 °C under 5% CO_2 for 1 h. After incubation at 37 °C 5% CO_2 for 24 h, the cells form three groups were stained with 1% crystal violet. The optical density of the cellular lysate after treatment with 1% SDS was measured at a wavelength of 590 nm. Each concentration took up three wells during each assay, three independent determinations were carried out.

3.3. Hemagglutination (HA) inhibition activity

To study the antiviral mechanism of TF derivatives, inhibition of HA was evaluated. The HA inhibition assay results (Table 3) showed that the TF80%, TF-3-G, or TF-3′-G could completely inhibit viral binding to guinea pig erythrocytes for both A/PR/8/34(H1N1) and A/Sydney/5/97(H3N2) strains with the minimum effective concentration (MEC) of ⩽25 μg/mL, while TF-3,3′-DG showed no inhibitory effect on HA from H3N2. For the B/Jiangsu/10/2003 strain, only TF-3-G exhibited an inhibitory effect with MEC of 25 μg/mL. Additionally, TF-3-G showed the highest level of HA inhibition against both A/PR/8/34(H1N1) and A/Sydney/5/97(H3N2), with a MEC of 4.69 ± 2.71 and 3.125 ± 0.00 μg/mL, respectively.

3.4. Inhibitory activity on influenza virus replication

To identify any inhibitory effect of TF derivatives on influenza virus replication, the synthesis of influenza viral HA mRNA was compared between TF treated and untreated infected cells. Total RNA extraction was performed at 7 h after influenza virus infection, and the levels of intracellular influenza mRNA were measured. After reverse transcription, a standard curve for the amplification of both the HA gene and reference GAPDH gene was generated by a serial 10-fold dilution of the template to evaluate the efficiencies of both reactions. The resulting standard curves showed that the amplification efficiencies for the genes were between 95% and 105% with correlation coefficients greater than 0.99. The results of the qPCR showed a significantly increased

Table 3
MEC values of HA inhibitory activity of TF derivatives.

Samples	Minimum effective concentration (MEC) (μg/mL)		
	A/PR/8/34(H1N1)	A/Sydney/5/97(H3N2)	B/Jiangsu/10/2003
TF80%	21.87 ± 5.41	14.58 ± 9.55	>50
TF-3-G	4.69 ± 2.71	3.125 ± 0.00	25.00 ± 0.00
TF-3′-G	13.80 ± 10.61	8.33 ± 3.61	>50
TF-3,3′-DG	13.15 ± 11.54	>135.42	>50

level of HA mRNA transcription in the inoculated cells at 7 h after viral infection compared to the mock. Also, a reduction of HA mRNA transcription was observed in cells treated with the 80%TF, TF-3-G, TF-3′-G, or TF-3,3′-DG compared with the non-treated infected cells in a dose-dependent manner using concentrations ranging from 1 to 10 μg/mL (Fig. 3).

3.5. Influence on vRNP localization in the nucleus

Typically, the viral ribonucleoprotein (vRNP) is confined to the nucleus in the early stage of infection, but is transported to the cytoplasm for packaging into progeny virions in the late stage of infection. To investigate the influence of three TF derivatives on the localization of the influenza virus vRNP in the nucleus of infected cells, A549 cells were co-treated with virus and the TF derivatives in concentrations of 3, 10 or 30 μg/mL. At 12 h post-infection, cells were analyzed for vRNP localization by immunofluorescence. Compared to untreated infected cells, all the TF derivatives remarkably decreased the nuclear localization of vRNP in a dose-dependent manner as demonstrated by immunofluorescence staining and inhibition of influenza virus replication (Fig. 4).

3.6. Influence on cytokine production in H1N1-infected cells

The virus-induced "cytokine storm" appears to contribute to the severe pathogenesis of the reconstructed 1918 H1N1 and H5N1 influenza viruses pandemics (Beigel et al., 2005; de Jong et al., 2006; Kash et al., 2006); therefore, the inhibition of virus-induced cytokine release is also important for the treatment of influenza. In addition to investigating inhibitory effects against the virus, the influence of derivatives on the production of cytokines that have been closely linked to the progression of H1N1 infections was studied. Supernatants from cultures treated or untreated with TF derivatives were compared for the expression level of IL-6 by ELISA (Fig. 5). Mock-infected cells maintained a level of IL-6 as low as 16.76 ± 8.2 pg/mL, while H1N1 infection dramatically increased the level of IL-6 by 21.1-fold (352.9 ± 17.7 pg/mL). Treatment with TF derivatives did not significantly alter basal cytokine level of normal cells at concentrations up to 25 μg/mL. The H1N1-induced cytokine secretion was significantly reduced by TF derivatives in a dose-dependent manner compared with the untreated H1N1-infected cells.

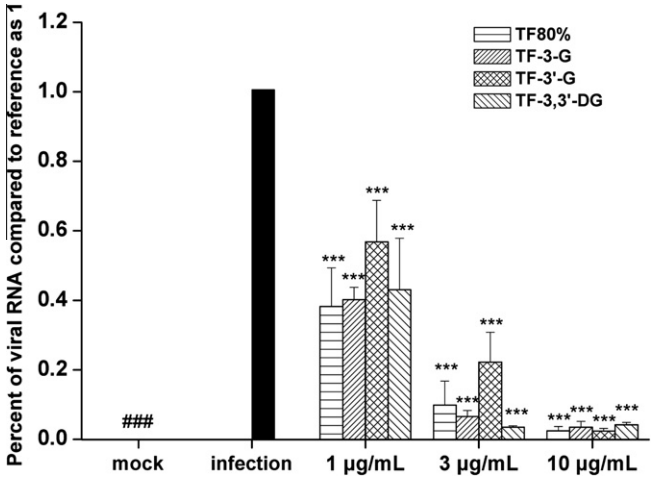

Fig. 3. qPCR of influenza viral RNA levels normalized to GAPDH. A549 cells were infected by influenza virus (MOI 0.1) at the same time as treatment with three TF derivatives (1, 3 or 10 μg/mL). Total RNA extraction was performed at 7 h after infection. After reverse transcription, the levels of intracellular influenza viral HA mRNA were measured by qPCR. The $2^{-\Delta\Delta Cq}$ (Livak) algorithm was used to analyze the C_q values based on the approximate efficiencies of the target gene and reference gene. Influenza viral HA mRNA levels were normalized to GAPDH as 1. In all, three biological replicates and three technical replicates were performed with similar results ($^{\#\#\#}p < 0.01$; $^{***}p < 0.01$).

4. Discussion

The influenza virus life cycle begins with viral HA binding to sialylated glycoprotein receptors on the host cell surface, followed by receptor-mediated endocytosis. The virus enters the cell by internalization. Subsequently, endosomal acidification alters the conformation of HA and leads to the fusion of the host and viral membranes, which permits the release of the viral ribonucleoproteins (vRNPs) into the cytoplasm. In the nucleus of infected cells, the viral RNAs are transcribed into mRNAs and replicated. Finally, the newly synthesised vRNPs are exported into the cytoplasm, and after packaging, mature virions are released from the cell surface which is dependent on sialidase cleaving sialic acid (SA) receptors (Min and Subbarao, 2010). TF-3,3′-DG has been reported to prevent the virus from being adsorbed into MDCK cells, which directly inhibits the hemagglutinin of the influenza virus, as observed with an electron microscope (Nakayama et al., 1993). However, the knowledge of the anti-influenza virus activity of TF-3,-3′-DG and their derivatives, and their potential mechanisms are still limited. Four probable pathways might be deduced for their antiviral effects: (1) blockage of viral binding to the cell receptors in the early stage of a virus infection; (2) attenuation of viral replication after entry; (3) inhibition of virus release from host cell; and (4) reduction of the serious inflammatory cytokines storm induced by the influenza virus infection.

During the time-of-addition assays, the antiviral effects with pre-incubation treatment, simultaneous treatment and post-treatment were compared to identify whether TF derivatives could block viral entry. The CPE reduction assay results showed higher inhibitory activity with the simultaneous treatment for each drug than post-infection treatment. Moreover, to corroborate the anti-influenza virus activity in the simultaneous treatment assay, a qPCR assay was used to test the *in vitro* effect of these drugs on viral replication. Furthermore, the results from the HA inhibition assay suggest that TF derivatives could inhibit HA by directly binding to viral HA or by blocking the SA receptor on the cell surface, which would be consistent with the assay results reported in the literature (Nakayama et al., 1993). Hence, it is strongly indicated that blockage of viral adsorption to the host cell might be one of the mechanisms for their antiviral activity. The CPE, qPCR and HA inhibition experimental results indicated that TF derivatives could disturb viral entry. In addition, the results using immunofluorescence staining for NP as a marker of vRNP localization revealed that the inhibition by three TF derivatives on vRNP synthesis and localization in the nucleus might be an indirect downstream effect of blocked entry.

In addition, we first reported the inhibitory effects of TF derivatives on neuraminidase of three different subtypes of influenza virus strains, which are in accordance with the results from the CPE reduction assay of the post-treatment. Furthermore, both NA and HA recognize carbohydrate structures and bind to sialic acid units on the surface of host cell. By cleaving the α-(2,6) glycosidic linkage, NA reduces the number of receptor binding sites for HA on host cells and progeny viruses, which allows the mature virus to be released from the host cell (Manco et al., 2006). The NA activity assay results showed that the mild effect of TF derivatives against influenza viral neuraminidase was crucial for their antiviral activity in the late stage when the virus detached from the host cells during release, although their potency was much poorer than that of oseltamivir carboxylate.

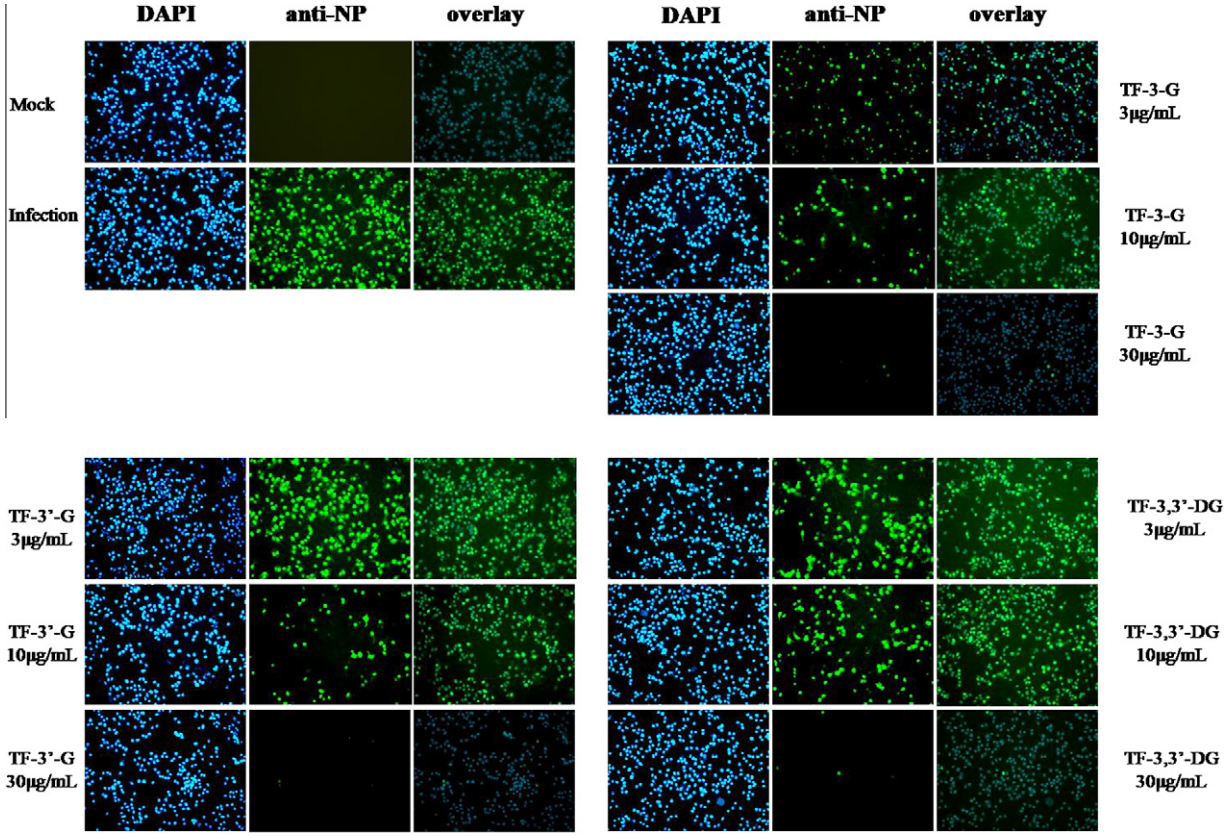

Fig. 4. TF derivatives treatment on the vRNP localization in the nucleus of H1N1-infected A549 cells. TF-3-G, or TF-3′-G, or TF-3,3′-DG at 3, 10 or 30 μg/mL were added with influenza virus infection A/PR/8/34 (H1N1) at MOI 0.1. Twelve hours post-infection, NP localization was visualized using specific antibodies with an immunofluorescence microscope. NP staining is shown in green. Nuclei stained by DAPI is shown in blue. Photographs were taken randomly from one representative experiment. In total, three independent experiments were performed with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

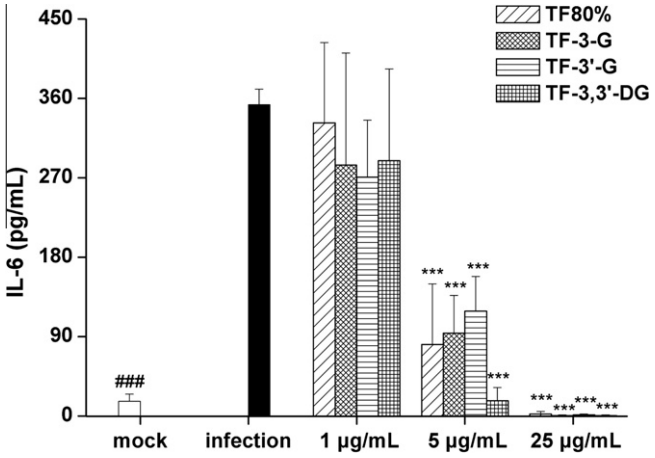

Fig. 5. Influence of TF derivatives treatment on the production of IL-6 in H1N1-infected A549 cells. The TF80%, TF-3-G, or TF-3′-G, or TF-3,3′-DG at 1, 5 or 25 μg/mL was added with influenza virus infection A/PR/8/34(H1N1) at MOI 0.1. At 24 h post-infection, the level of IL-6 in the supernatants was measured by ELISA. Data were represented as the mean ± SD of three separate experiments. $^{###}p < 0.01$, $^{***}p < 0.01$ relative to the untreated, virus-infected control.

As far as we know, polyphenolic compounds belong to one of the most important secondary metabolites in plant species. Various polyphenolic compounds have been studied for their NA inhibiting properties, among which, flavonoids and (oligo) stilbenes are two major structure types. Some sub-groups of flavonoids have been studied for their NAIs with a classification of their IC_{50} values into three groups of good, moderate and weak. Substances belonging to aurones have been investigated as most potent NAIs among flavonoid derivatives (IC_{50} values between 22.0 and 72.0 μM, H1N1) with essential functionalities of a hydroxyl group in position 6, an oxo group in position 3, and a double bond between position 2 and phenylidene moiety (Liu et al., 2008a,b). Biflavonoids like ginkgetin, hinokiflavone and 4′-*O*-methylochnaflavone with IC_{50} values between 2.01 and 97.09 μM (H1N1). Fourteen C-methylated flavonoids were found to have NA inhibiting properties (IC_{50}s 2.55–28.12 μM); among the chalcones, the coumestan glycyrol (IC_{50} 3.1 μM) and isoliquiritigenin (IC_{50} 9.0 μM) have been studied; flavones have also proven significant activities against influenza NA with IC_{50}s (25.92–33.71 μM, H1N1) and (2.2–8.8 μM, recombinant H1N1). As isoflavones, daidzein (IC_{50} 37.1 μM, H1N1) and genistein (IC_{50} 77.1 μM, H1N1) displayed inhibitory activities against NA. On the other hand, some (oligo) stilenes were reported to perform antiviral activities by targeting influenza NA, with IC_{50} values from 8.94 to 61.16 μM (H1N1) (Liu et al., 2010; Grienke et al., 2012). For flavonoids, it was reported that the position and number of hydroxyl groups, especially in position 4′ and 7, an oxo group in position 4, and a double bond between position 2 and 3 are essential for good NA inhibitory activity (Liu et al., 2008a,b). In the present study, as a subtype of polyphenols in black tea, theaflavin and its derivatives containing a benzo tropolone ring and two catechins with or without catechin gallates, showed inhibitory effect against NA although catechin as a polyphenolic compound showed no inhibition on NA. The studies indicated that the scaffold of TFs is in favor of NA inhibition, and two gallates as substitutes or functional groups at 3 and 3′ positions are more favorable for activity than one at 3 or 3′

position. So we speculate that the NA inhibition of polyphenols depend on both their scaffolds and substituents or functional groups.

It was concluded that TF derivatives may act as a potent anti-influenza virus agent primarily at an early stage during the infection. Typically, vRNP is confined to the nucleus in the early stage of infection, but it is exported to the cytoplasm in the late stage. In our experiment, NP was used as a marker of vRNP localization by immunofluorescence staining (Josset et al., 2008). The result showed potent inhibitory effect of TF derivatives against influenza virus vRNP nucleus localization at in dose-dependent manner without apparent cytotoxicity. There have been data indicating that knockdown of 11 host cell genes including NXF1, COPG, SON, and ATP6V0C seemed to reduce NP expression and nuclear export by siRNA transfection and immunofluorescence staining (Karlas et al., 2010). Interestingly, most of the analyzed genes had no effect on viral entry, as robust vRNA still could be detected after gene silencing. Furthermore, most of these genes are related to RNA biogenesis and viral mRNA synthesis. We have reason to speculate that besides the inhibitory effect on virus entry, the theaflavins extract and compounds may affect virus reproduction by down-regulating the host cell genes at a stage between virus entry and mRNA synthesis.

Finally, apoptosis might be a direct consequence of both viral replication and the excessive inflammatory responses to virus infection. Production of reaction oxygen species (ROS) in leukocytes and epithelial cells may be involved in the pulmonary damage caused by virus infection (Oda et al., 1989). Endogenous oxidants may stimulate production of cytokines through the activation of transcription factors and induction of pro-inflammatory gene expression in influenza A virus-infected cells (Knobil et al., 1998). The assay results showed that TF derivatives could significantly reduce H1N1 virus-induced cytokine IL-6 secretion, which indicated that they also possess an anti-inflammatory effect.

Therefore, we conclude that TF derivatives possess both anti-influenza virus and anti-H1N1-induced-inflammation activities. Although we still lack *in vivo* experimental data, the activity study of TFs on influenza and underlying mechanisms will provide a theoretical and experimental basis to guide novel anti-influenza virus drug discovery.

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