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Inhibition of influenza virus replication by plant-derived isoquercetin

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ABSTRACT

Influenza virus infects the respiratory system of human and animals causing mild to severe illness which could lead to death. Although vaccines are available, there is still a great need for influenza antiviral drugs to reduce disease progression and virus transmission. Currently two classes (M2 channel blockers and neuraminidase inhibitors) of FDA-approved influenza antiviral drugs are available, but there are great concerns of emergence of viral resistance. Therefore, timely development of new antiviral drugs against influenza viruses is crucial. Plant-derived polyphenols have been studied for antioxidant activity, anti-carcinogenic, and cardio- and neuroprotective actions. Recently, some polyphenols, such as resveratrol and epigallocatechin gallate, showed significant anti-influenza activity in vitro and/or in vivo. Therefore we investigated selected polyphenols for their antiviral activity against influenza A and B viruses. Among the polyphenols we tested, isoquercetin inhibited the replication of both influenza A and B viruses at the lowest effective concentration. In a double treatment of isoquercetin and amantadine, synergistic effects were observed on the reduction of viral replication in vitro. The serial passages of virus in the presence of isoquercetin did not lead to the emergence of resistant virus, and the addition of isoquercetin to amantadine or oseltamivir treatment suppressed the emergence of amantadine- or oseltamivir-resistant virus. In a mouse model of influenza virus infection, isoquercetin administered intraperitoneally to mice inoculated with human influenza A virus significantly decreased the virus titers and pathological changes in the lung. Our results suggest that isoquercetin may have the potential to be developed as a therapeutic agent for the treatment of influenza virus infection and for the suppression of resistance in combination therapy with existing drugs.

1. Introduction

Influenza viruses (currently circulating H1N1 and H3N2) cause 3–5 million cases of severe illness and up to 500 000 deaths worldwide per year (Russell et al., 2008). In the U.S. there are approximately 36 000 deaths and over 200 000 hospitalizations due to influenza-related complications each year (Thompson et al., 2003). Influenza viruses are highly diverse with antigenic drift and shift, which could lead to emergence of viruses that have not been exposed to human population, leading to pandemic outbreaks. Because of the importance of influenza virus infection, there have been major efforts in the development of effective antivirals for the last several decades (Palese and Shaw, 2007). There are now four U.S. FDA-approved drugs on the market; amantadine, rimantadine, oseltamivir, and zanamivir (Palese and Shaw, 2007). Amantadine and rimantadine target the M2 ion channel of influenza virus to prevent viral uncoating. Also they affect the pH regulation of vesicles in which viral glycoproteins are transported to cytoplasm for assembly (Palese and Shaw, 2007). The other classes of antiviral drugs are neuraminidase (NA) inhibitors, oseltamivir and zanamivir. Influenza A and B viruses bind to the sialic acid glycoconjugates on the host cell by their hemagglutinin (HA) molecules (Palese and Shaw, 2007). After successfully penetrating and replicating in the cells, neuraminidase (NA) cleaves the host cell sialic acid glycoconjugates and the mature viruses bud from the cell surface. The NA inhibitors inhibit the cleavage of sialic acid residues, preventing the release of the viruses (Palese and Shaw, 2007).

Currently all the FDA-approved drugs target viral proteins and have been associated with emergence of viral resistance (MMWR, 2009; Poland et al., 2009). The M2 blocking antivirals have been well documented for their ability to raise resistant variants. Early in the 2005/2006 flu season, 92.3% of influenza A viruses analyzed from 26 states in the U.S. contained point mutations that conferred amantadine resistance (Bright et al., 2006). Therefore, only the NA inhibitors remain effective against the currently circulating viruses. However, recent reports suggested that most circulating influenza viruses during 2007–2008 seasons in the U.S. were also resistant to oseltamivir (Dharan et al., 2009). In 2009, a H1N1 pandemic occurred in April and spread across the world (Garten et al., 2009). As of November 2009, there were already 57 cases of oseltamivir-resistant viruses worldwide, indicating

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again that antiviral resistance arises quickly with drug use (WHO, 2009).

There are numerous studies on the beneficial effects of polyphenols from plants and fruits in humans and animals. Quercetin is present in various plants and particularly abundant in onions and tea, and exists predominantly in glucoside forms including isoquercetin (Lakhanpal and Rai, 2007; Scalbert and Williamson, 2000). Some polyphenols also showed antiviral effects. Resveratrol and quercetin reported to inhibit the replication of various viruses (Chiang et al., 2003; Debiaggi et al., 1990; Docherty et al., 1999, 2006; Heredia et al., 2000; Kaul et al., 1985), including influenza virus (Furuta et al., 2007; Mori et al., 2008; Nakayama et al., 1993; Song et al., 2005; Xiao et al., 2008).

In our study, we investigated selected polyphenols for their antiviral activity against influenza A and B viruses. Among the polyphenols we tested, isoquercetin had the lowest effective concentration against both influenza A and B viruses. In the double treatment of isoquercetin and amantadine, synergistic effects were observed on the reduction of viral replication in the cells. Furthermore, the addition of isoquercetin to amantadine or oseltamivir treatment suppressed the emergence of amantadine- or oseltamivir-resistant viruses. In a mouse model of influenza virus infection, isoquercetin administered intraperitoneally to mice inoculated with mouse-adapted human influenza A virus significantly decreased the virus titers and pathological changes in the lung. To our knowledge, this is the first report that isoquercetin has antiviral effects against influenza virus. Our results suggest that isoquercetin may have the potential to be developed as a therapeutic agent for the treatment of influenza virus infection and for the suppression of resistance in combination therapy with existing drugs.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) or Vero cells were maintained in Minimum Essential Medium (MEM) containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Two different influenza A viruses from pigs (A/swine/OH/511445/2007 [H1N1], Oh7) and human (A/PR/8/34 [H1N1], PR8), and human influenza B virus (B/Lee/40) were used in this study. Human influenza A and B viruses were obtained from ATCC (Manassas, VA). The Oh7 strain (H1N1) is a triple human/avian/swine reassortant strain from pigs from Ohio county fair that infected both pigs and humans in August 2007 (Vincent et al., 2009). Trypsin treated with L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) was obtained from Sigma–Aldrich (St Louis, MO).

2.2. Compounds

Polyphenols including quercetin, isoquercetin (quercetin-3-glucoside), resveratrol, rutin (quercetin-3-rutinoside), quercetin-3-galactoside, quercetin-3-rhamnoside, kaempferol, luteolin, fisetin, catechin, epigallocatechin (EGC) and EGCG were purchased from Sigma–Aldrich or ChromaDex Inc. (Irvine, CA). Oseltamivir carboxylate and amantadine were purchased from Fisher Scientific (Palatine, IL) and Sigma–Aldrich, respectively. Alcohol extracts of Hypericum perforatum and Equisetum arvense L were purchased from commercial sources. Each compound was dissolved in dimethyl sulfoxide (DMSO), and 0.1% DMSO was used as a negative control.

2.3. Nonspecific cytotoxic effects by the compounds

We determined the toxic dose for 50% cell death (TD_{50}) for each compound in MDCK and Vero cells. Confluent MDCK or Vero cells grown in 96-well plates were treated with various concentrations (1–200 μM) of each compound for 24–48 h. Cell cytotoxicity was measured by a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI) and crystal violet staining.

2.4. Screening of the compounds for anti-influenza virus activity

Virus infection was performed as follows. Two-day-old monolayers of MDCK cells or Vero cells were prepared in 6-well plates. Influenza virus (Oh7, PR8 and B/Lee/40) was inoculated to the cells at a multiplicity of infection (MOI) of 0.05 or 5, and the virus-infected cells were incubated in the presence of each compound at various concentrations (0.1–150 μM) with trypsin (10 μg/ml) for up to 4 days. Amantadine or oseltamivir carboxylate served as positive controls. Virus replication was assessed with various methods including immunofluorescence assay (IFA), Western blot analysis, and virus titration with $TCID_{50}$ method, and real-time qRT-PCR.

2.4.1. IFA

Virus-infected cells were fixed with 100% methanol for 15 min and rinsed with phosphate buffered saline (PBS). The cells were treated with blocking buffer (PBS with 2% bovine serum albumin) for 1 h, and primary antibody (NP-specific goat-antiserum, Santa Cruz Biotech) was added to the cells overnight. Following rinsing with PBS, specific secondary antibody with fluorescent probe (FITC-coupled anti-goat IgG) was added to the cells for 2 h. Fluorescence was detected by fluorescence microscopy.

2.4.2. Western blot analysis

Cell lysates from MDCK or Vero cells were prepared by adding sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% β-mercaptoethanol and sonication for 20 s. Then the proteins were resolved in a 10% Novex Bis-Glycin gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The transferred nitrocellulose membranes were incubated with primary antibodies to influenza viruses (whole virions) (Santa Cruz Biotech, Santa Cruz, CA) or β-actin as a loading control overnight, and then with the secondary antibodies conjugated with peroxidase for 2 h. Following incubation with a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce biotechnology, Rockford, IL), the signals were detected on X-ray film.

2.4.3. $TCID_{50}$ method

A standard $TCID_{50}$ method with the 10-fold dilution of each sample was used for virus titration (Marjuki et al., 2009; Reed and Muench, 1938).

2.4.4. Real-time qRT-PCR

The viral RNAs were extracted from cells or tissues with the RNeasy kit according to the manufacturer's directions (Qiagen, Valencia, CA). Virus specific primers and probe were synthesized based on literature (Schweiger et al., 2000). The primer sequences for influenza A virus M gene were: Forward 5′-CATGGAATGGCTAAAGACAAGACC-3′ and reverse 5′-AAGTGCACCAGCAGAATAACTGAG-3′. The probe sequence used was: FAM-5′-CTGCAGCGTAGACGCTTTGTCCAAAATG-3′-Iowa Black. Using the One-step Platinum qRT-PCR kit (Invitrogen, Carlsbad, CA), the qRT-PCR amplification was performed in a SmartCycler (Cepheid, Sunnyvale, CA) with the following parameters: 45 °C for 30 min, and 95 °C 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and elongation at 72 °C for 30 s.

2.5. Antiviral effects of Hypericum perforatum (St. John's Wort) and Equisetum arvense L (Field horsetail) against influenza virus

It was previously shown that the extracts of Hypericum perforatum and Equisetum arvense L contain high amounts of isoquercetin (Kazlauskas and Bagdonaite, 2004; Li and Fitzloff, 2001; Mimica-Dukic et al., 2008; Tatsis et al., 2007). We obtained the alcohol extracts of Hypericum perforatum and Equisetum arvense L from commercial sources to examine their effects on the replication of influenza virus. First, we analyzed the presence of isoquercetin in these extracts using high-performance liquid chromatography (HPLC) analysis. HPLC was done with Shimadzu 10ATvp UV/vis HPLC system after each sample was diluted in 75% ethanol (1:5) and filtered through 0.22 μm membrane filters. Analysis was carried out at room temperature on a Waters YMC ODS-AQ RP-18 column (Waters, Milford, MA). The mobile phases consisted of water (containing 20% methanol and 0.5% trifluoroacetic acid [TFA]) and acetonitrile (containing 10% methanol and 0.5% TFA). The analysis was performed with a linear gradient program. The injection volume was 10 μl and the flow-rate was kept at 1 ml/min to 60 min. We used isoquercetin and quercetin (both >95%) as controls for HPLC analysis. To examine the antiviral effects on influenza virus, each extract was further diluted in ethanol up to 1:4000. Confluent MDCK cells were inoculated with Oh7 strain at a MOI of 0.05, and each extract was added to the medium at dilutions of 1:500, 1:1000, 1:2000, and 1:4000. The virus replication was measured by $TCID_{50}$ assay, and statistical analyses were done using Student's *t*-test ($P < 0.05$).

2.6. The combination treatment of isoquercetin and amantadine or oseltamivir

The combination treatment of isoquercetin and amantadine was performed to demonstrate additive or synergistic effects on the replication of influenza virus. The Oh7 strain was inoculated in MDCK cells at a MOI of 5 or 0.05 for 1 h, and then the medium was replaced with mock-medium, or medium containing amantadine (5 or 1 μM), isoquercetin (2 or 0.5 μM), or a combination of isoquercetin (2 or 0.5 μM) and amantadine (5 or 1 μM). The infected cells were further incubated for 24 or 48 h, and virus replication was measured by $TCID_{50}$ assay. Drug–drug interactions were analyzed by the three-dimensional model of Prichard and Shipman (1990), using the MacSynergy II software at 95% confidence limits. Theoretical additive interactions were calculated from the dose–response curve for each compound individually, and the calculated additive surface was subtracted from the experimentally determined dose–response surface to give regions of synergistic or antagonistic interactions. The resulting surface appears as horizontal plane at 0% of synergy if the interactions of two compounds are additive. Any peak above or below this plane indicates synergy or antagonism, respectively.

In separate experiments for viral resistance, one-day-old MDCK cells in 6-well plates were infected with Oh7 strain at a MOI of 5 or 0.05 for 1 h, and isoquercetin (2 μM), amantadine (2 μM), or oseltamivir carboxylate (2 μM) was added to each well. The supernatants were collected every two days and used for titration ($TCID_{50}$) and infecting newly prepared MDCK cells up to 10 passages. In the next set of experiments, isoquercetin (2 μM) was added to amantadine (2 μM) or oseltamivir carboxylate (2 μM) treatment, and the supernatant at each passage was used for titration for up to 10 passages.

2.7. Treatment of viruses and cells with isoquercetin

To investigate if isoquercetin has virucidal effects on influenza virus, influenza viruses were pre-incubated with isoquercetin. Influenza virus (Oh7) of high titer (>10^9 $TCID_{50}$/ml) was incubated with 1 or 5 μM of isoquercetin or DMSO at 37 °C for 2 h. Then the mixture was diluted (up to 100 times) before infection. Virus-infected cells were incubated with medium containing trypsin. The virus replication was monitored by the titration of progeny viruses with $TCID_{50}$ method or real-time qRT-PCR. We also pretreated MDCK cells with isoquercetin at 1–5 μM at 37 °C for 4 h and then inoculated the cells with influenza virus after washing the cells with PBS. In separate experiments, cells were inoculated with viruses, washed with PBS and incubated with medium containing trypsin at 37 °C for up to 48 h. During the incubation, isoquercetin (2 μM) was added to the cells at 0, 1, 2, 4, and 6 h after virus inoculation (1 MOI). For titration, virus-infected cells were freeze–thawed twice to ensure complete cell lysis. After centrifugation, the supernatant was used for $TCID_{50}$ assay in MDCK cells.

2.8. Mouse study

Four-week-old female BALB/c mice were housed and studied under Institutional Animal Care and Use Committee-approved protocols. Briefly, mice were purchased from Charles River Lab (Wilmington, MA) and randomly divided into five groups (5 mice/group). The mouse-adapted PR8 strain (A/PR/8/34, H1N1) (1 LD_{50}) was used to infect mice. Group 1 was infected with virus and did not receive treatment. Groups 2 and 3 were infected with virus and intraperitoneally (i.p.) received isoquercetin 2 mg/kg/day or 10 mg/kg/day, respectively. Group 4 received isoquercetin 10 mg/kg/day intraperitoneally for the duration of the study without virus inoculation. Group 5 was not infected with virus and did not receive isoquercetin.

The isoquercetin administration started 2 days prior to virus inoculation and continued daily for 6 days post-inoculation. On the day of virus inoculation, mice were lightly anesthetized with isofluorane and inoculated intranasally with 30 μl of influenza virus (approximately 3 hemagglutination unit [HAU]/mice, 1 LD_{50}) diluted in sterile saline. Isoquercetin was dissolved in DMSO and further diluted with PBS to a total volume of 100 μl (final concentration of DMSO was 10%). All mice were euthanized 6 days post-virus inoculation for lung harvest. The left lungs were collected to determine the virus titer and IFN-γ, RANTES and iNOS levels (as indication of lung inflammation), and the right lungs were fixed with 10% formalin for histopathology. The left lungs were homogenized in 2 ml of RLT with β-mercaptoethanol, and viral RNAs were extracted using RNeasy kit (Qiagen, Valencia, CA). Lung virus titers were determined by real-time qRT-PCR using primers for M protein (Schweiger et al., 2000). Likewise, mRNAs of IFN-γ, RANTES, and iNOS were detected by real-time qRT-PCR using Gene Expression Assay from Applied Biosystem (Foster City, CA). Statistical significance of the data was determined using Student's *t*-test. Nine-2 or 4-fold dilutions of stock virus (2×10^8 $TCID_{50}$/ml) were used for real-time qRT-PCR to generate a standard curve. The $\log_{10}$ titer of virus in a specimen was plotted against the Ct value, and a best-fit line was constructed with a correlation coefficient of 0.9914. Influenza virus quantity in the samples, expressed as $\log_{10}$ $TCID_{50}$/ml equivalents, is derived by plotting the Ct of a sample on the standard curve.

2.9. Histopathology

The right lung lobes were fixed in 10% buffered formaldehyde, embed in low-melting-point paraffin, and sectioned at 5 μm thickness. Tissue sections were stained with hematoxylin and eosin (Allen, 1992) and the microscopic lesions were observed in a double-blind fashion by a board-certified veterinary pathologist (Narayanan) to determine a histopathology score. The histopathol-

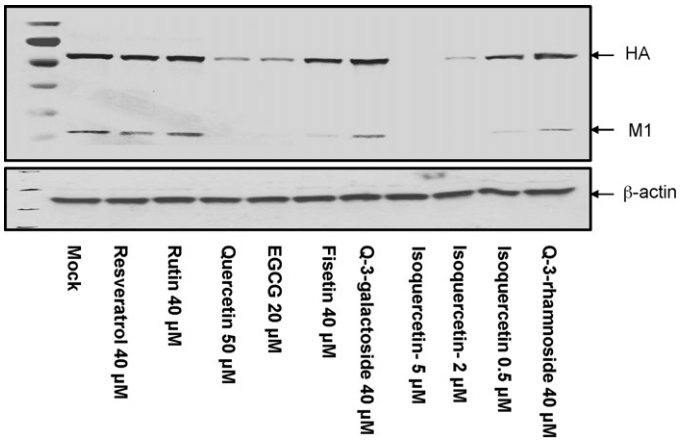

Fig. 1. Effects of various polyphenol compounds on the expression of influenza virus proteins in MDCK cells. Cell lysates were prepared after cells were infected with Oh7 strain at 5 MOI, and treated with mock-medium or various concentrations of each compound for 12 h. Western blot analysis of cell lysates was performed with antibodies against whole influenza virus or β-actin. Compounds include quercetin-3-rhamnoside (Q-3-rhamnoside, 40 μM), quercetin-3-galactoside (Q-3-galactoside, 40 μM), isoquercetin (0.5, 2 and 5 μM), fisetin (40 μM), EGCG (20 μM), quercetin (50 μM), rutin (40 μM), resveratrol (40 μM), and mock.

ogy scores were assigned between 0 (normal) to 5 (severe) based on five criteria: presence of necrotic bronchial and bronchiolar epithelium, serocellular exudates in the bronchial and bronchiolar lumina, inflammatory cells (predominantly neutrophils and lymphocytes) in the bronchiolar, peribronchiolar, and alveolar interstitium, peribronchiolar or alveolar collapse (atelectasis) and diffuse or multifocal interstitial edema.

3. Results

3.1. Inhibition of influenza viruses in MDCK or Vero cells by the compounds

The cells were infected with influenza A or B viruses and incubated in the presence of each compound at various concentrations (0.1–150 μM) with trypsin (10 μg/ml) for up to 4 days for the observation of cytopathic effects (CPE). The effective dose for 50% virus reduction (ED_{50}) and TD_{50} of each compound against various viruses were comparable in MDCK and Vero cells, and we used MDCK cells for detailed analysis. Virus replication was also confirmed by IFA (not shown) and Western blot analysis (Fig. 1) with antibodies to the viral nucleoprotein (NP) or whole influenza virus, respectively. While CPE progressed 12–18 h post-infection in the cells treated with 0.1% DMSO only (a negative control), there was a significant delay of CPE in the presence of quercetin, isoquercetin, resveratrol, kaempferol, luteolin, fisetin, and EGCG at different concentrations. However, there was little evidence for the reduction of CPE by catechin, quercetin-3-galactoside, quercetin-3-rhamnoside, rutin or EGC. The reduction of virus replication by the effective compounds was also evident with IFA staining or Western blot. The effects of each compound were similar against all viruses tested (Oh7, PR8, and B/Lee/40), suggesting broad-effectiveness of these compounds. PR8 strain is naturally resistant to amantadine and there was no effect of amantadine on the replication of PR8 strain and influenza B virus (B/Lee/40) as expected. However, the progress of CPE from Oh7 strain was delayed by amantadine (ED_{50} approximately 1.4 μM). Oseltamivir carboxylate was effective against all virus strains tested. The TD_{50} was determined at 24 h of incubation of each compound in MDCK cells using CytoTox 96® non-radioactive cytotoxicity assay kit and crystal violet staining (Table 1). For the determination of ED_{50}, virus-infected MDCK cells were treated with increasing concentrations of each compound (up to 150 μM), amantadine or oseltamivir carboxylate (up to 50 μM) with trypsin for 24 h, and virus titers were determined by $TCID_{50}$ method. The TD_{50} and ED_{50} of each compound, amantadine and oseltamivir against Oh7 strain in MDCK cells at 24 h were

Table 1
Effects of the selected polyphenols on the replication of influenza A virus (Oh7 strain). The effective dose for 50% reduction on viral replication (ED_{50}) and the toxic dose for 50% cell death (TD_{50}) of each compound, amantadine and oseltamivir carboxylate were determined at 24 h of incubation in MDCK cells.

Compound	ED_{50} (μM)	TD_{50} (μM)	In vitro therapeutic index
EGCG	8	45	5.6
Resveratrol	45	90	2
Fisetin	57	86	1.5
Quercetin	48	83	1.7
Rutin	>100	>200	–
Isoquercetin	1.2	46	38
Amantadine	1.4	180	129
Oseltamivir	0.5	>200	>400

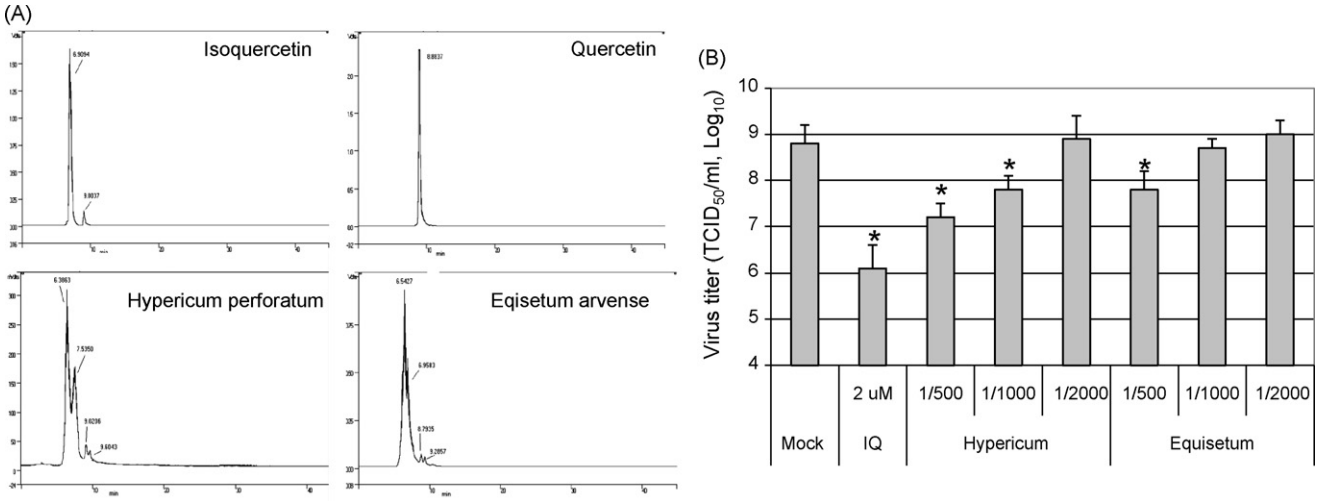

Fig. 2. HPLC analysis of extracts of Hypericum perforatum and Equisetum arvense L and their effects on influenza virus replication. (A) HPLC analysis of isoquercetin and quercetin (upper panel) and the extracts (lower panel). (B) Antiviral effects of isoquercetin (IQ, 2 μM), Hypericum perforatum extract (Hypericum, 1:500 to 1:2000) and Equisetum arvense L extract (Equisetum, 1:500 to 1:2000). Virus replication in the presence of mock-medium or each compound was measured by the titration of progeny virus after 24 h. *Statistically significant ($p < 0.05$) compared to virus-infected cells without treatment by Student's *t*-test.

Table 2
Effects of combinations of isoquercetin and amantadine on influenza A virus infection at 24 h of incubation in MDCK cells.

Drug	Concentration (μM)	Virus[a] titers with[b]		
		Isoquercetin (μM)		
		0	2	0.5
Amantadine	0	8.30 ± 0.14	6.55 ± 1.76	8.20 ± 0.42
	5	6.30 ± 0.98	**4.95 ± 0.63**	**5.6 ± 0.70**
	1	7.95 ± 0.21	**5.05 ± 1.06**	**7.1 ± 0.42**

[a] Influenza A/swine/OH/511445/2007 [H1N1], Oh7 strain.
[b] Values are means ± standard deviations of virus titers (log_{10} $TCID_{50}$/ml). Boldface values indicate regions of synergy.

Table 3
Effects of isoquercetin on the emergence of viral resistance to amantadine and oseltamivir in serial viral passages. The ED_{50} to each compound was measured at each passage with the single or combination treatment at the indicated concentration.

Passage number	Isoquercetin (2 μM)	Amantadine (2 μM)	Amantadine (2 μM) + isoquercetin (2 μM)	Oseltamivir (2 μM)	Oseltamivir (2 μM) + isoquercetin (2 μM)
P0	1.2	1.4	1.4	0.5	0.5
P1	1.2	5.5	1.4	0.5	0.5
P2	1.2	10.4	1.4	0.5	0.5
P3	1.2	>50	1.4	2.3	0.5
P5	1.2	>50	1.4	5.5	0.5
P10	1.2	>50	1.4	>20	0.5

determined and shown in Table 1. The in vitro therapeutic index was calculated by dividing TD_{50} by ED_{50}. EGCG had ED_{50} of 8 μM and TD_{50} of 45 μM. Resveratrol, fisetin, and quercetin had ED_{50} of 45–57 μM with low in vitro therapeutic index. It has been reported that resveratrol and EGCG have anti-influenza activity in vitro (and in vivo for resveratrol), and ED_{50} for these compounds in our study were similar to those in the literature (Davis et al., 2008; Furuta et al., 2007; Mori et al., 2008; Nakayama et al., 1993; Palamara et al., 2005; Song et al., 2005). Among the polyphenols tested, isoquercetin had the lowest ED_{50} against both influenza A and B viruses with a higher in vitro therapeutic index. For Oh7 strain, ED_{50} and TD_{50} of isoquercetin were 1.2 μM and 46 μM, respectively (Table 1). Quercetin, isoquercetin, resveratrol, kaempferol, luteolin, fisetin, and EGCG were also effective to all influenza A strains we tested as well as influenza B virus in MDCK cells. The ED_{50} of each compound against PR8 strain and influenza B virus were similar to that against the Oh7 strain.

3.2. The effects of extracts of Hypericum perforatum (St. John's Wort) and Equisetum arvense L (Field horsetail) on the replication of influenza virus

Both extracts contained abundant amounts of isoquercetin, but not quercetin, on HPLC analysis (Fig. 2 A), and significantly reduced the titer of influenza virus by up to 20-fold at 1:500 (Equisetum arvense L) or 1:1000 dilutions (Hypericum perforatum) at 24 h post-inoculation (Fig. 2B). These results confirmed the antiviral effects of isoquecetin in pure (>95%) or crude forms from various plants.

3.3. The combination treatment of isoquercetin and amantadine or oseltamivir

The combination treatment of isoquercetin and amantadine was performed to demonstrate additive or synergistic effects on the replication of influenza virus. When virus-infected cells were incubated in the presence of combinations of isoquercetin (2 or 0.5 μM) and amantadine (5 or 1 μM), synergistic effects were observed with a maximal synergy at lower concentrations of isoquercetin (0.5 μM) and amantadine (1 μM). The increasing concentrations of isoquercetin and amantadine showed decreasing synergism (Fig. 3, Table 2).

To determine if isoquercetin treatment leads to the emergence of viral resistance, viruses were passaged in the presence of isoquercetin. During the 10 passages of Oh7 strain in the presence of isoquercetin (2 μM), ED_{50} of isoquercetin did not increase, suggesting that isoquercetin did not induce resistant viruses. On the contrary, oseltamivir or amantadine treatment induced resistant viruses rapidly (Table 3). Since the combination treatment of amantadine and oseltamivir was reported to suppress viral resistance during viral passages in cells, isoquercetin was added to amantadine or oseltamivir treatment to determine if isoquercetin was able to suppress the emergence of resistant viruses to amantadine or oseltamivir. When isoquercetin (2 μM) was added to amantadine or oseltamivir (2 μM) in MDCK cells infected with viruses, resistance to either compound did not emerge up to 10 passages and virus

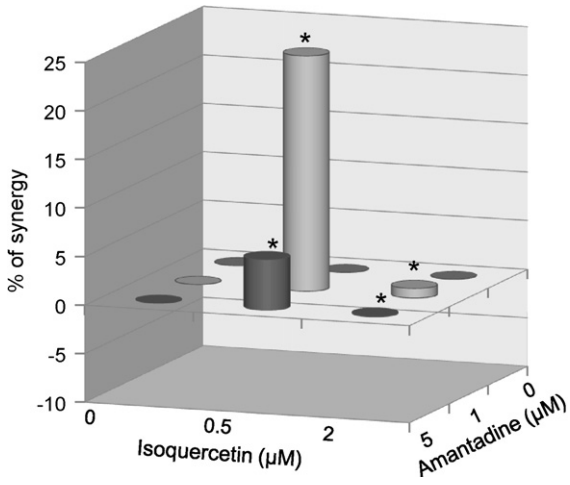

Fig. 3. Three-dimensional plots showing the interaction of isoquercetin and amantadine on the replication of influenza A/swine/OH/511445/2007 (Oh7 strain). The Oh7 strain was inoculated in MDCK cells at a MOI of 5 for 1 h, then the medium was replaced with mock-medium, or medium containing amantadine (5 or 1 μM), isoquercetin (2 or 0.5 μM), or combinations of amantadine (5 or 1 μM) and isoquercetin (2 or 0.5 μM). The cells were incubated for 24 h, and virus replication was measured by $TCID_{50}$ assay. Drug–drug interactions were analyzed by the three-dimensional model of Prichard and Shipman, using the MacSynergy II software at 95% confidence limits. Bars (*) above the plane of 0% synergy in the plot indicate synergy. The synergy was maximal at a combination of low concentrations of isoquercetin (0.5 μM) and amantadine (1 μM).

Table 4
Effects of isoquercetin on influenza A virus replication and expression of IFN-γ, RANTES, and iNOS in the lungs of mice.

Group	Virus titer (Ct) ($TCID_{50}$/ml)	IFN-γ (Ct)	RANTES (Ct)	iNOS (Ct)
Virus[a]	15.3 ± 1.4 ($7.5 \times 10^4 \pm 7.0 \times 10^4$)	21.7 ± 0.7	19.8 ± 0.6	22.2 ± 0.6
Virus + isoquercetin (2 mg/kg/day)	16.7 ± 0.6 ($2.3 \times 10^4 \pm 1.4 \times 10^4$)	22.8 ± 0.6*	21.5 ± 0.8*	23.9 ± 0.9*
Virus + isoquercetin (10 mg/kg/day)	19.6 ± 1.3* ($3.9 \times 10^3 \pm 2.9 \times 10^3$)	23.3 ± 0.8*	22.4 ± 0.9*	25.1 ± 0.6*
Isoquercetin (10 mg/kg/day)	–	30.0 ± 0.6	27.2 ± 0.3	28.0 ± 0.8
Mock (medium)	–	28.9 ± 1.2	25.0 ± 1.3	27.0 ± 1.8

The data represent the mean Ct or $TCID_{50}$/ml values ± standard deviations.
[a] Mouse-adapted human influenza A virus [A/Puerto Rico/8/34, PR8 (H1N1)].
* Statistically significant ($p < 0.05$) compared to untreated control group (virus-infected) by Student's *t*-test, $p < 0.05$.

titers gradually decreased to none, indicating the co-treatment completely inhibited the replication of viruses over time.

3.4. Treatment of viruses and cells with isoquercetin

The preincubation of influenza virus with isoquercetin did not decrease the viral titer, indicating that the antiviral effects of isoquercetin are not associated with direct viral neutralizing or virucidal effect on influenza virus. The preincubation of MDCK cells with isoquecetin (2 μM) for 4 h before virus infection following extensive washing with PBS significantly decreased the virus titers. Furthermore, the addition of isoquercetin up to 4 h post-infection (0, 1, 2, and 4 h) significantly decreased viral titers in a time-dependent manner, but not after 6 h following virus inoculation (Fig. 4).

3.5. The effects of isoquercetin on the replication of influenza A virus in a mouse model

The virus titers in the lungs decreased significantly in the isoquercetin treated group (10 mg/kg/day) compared to the untreated group by 4.34 CTs which corresponds to approximately 19-fold decrease in $TCID_{50}$/ml titers (Table 4, Fig. 5). The group treated with isoquercetin at 2 mg/kg/day had moderately reduced virus titers compared to the untreated group. In addition, isoquercetin treatment at 2 or 10 mg/kg/day significantly reduced the levels of IFN-γ, iNOS and RANTES in the lungs compared to the untreated group. Microscopic evaluation demonstrated that the bronchial and bronchiolar epithelia were necrotic in mice infected with Influenza virus, whereas, mice treated with isoquercetin following infection had intact epithelia, indicating that isoquercetin treatment is associated with less severe degree of viral bronchitis or bronchiolitis. The histopathology score of the lungs was lower in virus-infected mice treated with isoquercetin at 10 mg/kg/day compared to that of no treatment group, which correlates with our finding of significantly reduced viral titer and IFN-γ/iNOS/RANTES levels in the lungs of mice treated with isoquercetin (Tables 4 and 5, Fig. 6). The infected mice lost weight, but no mortality was observed for the duration of the study.

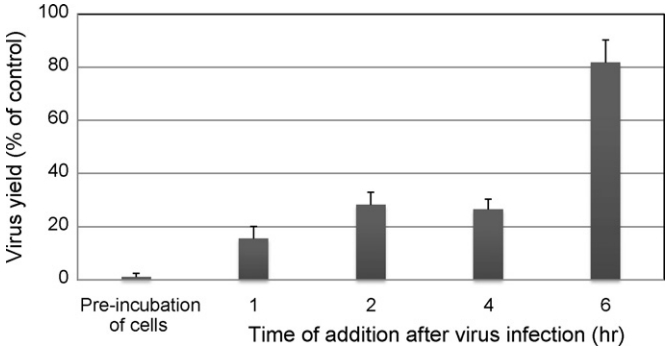

Fig. 4. Time of addition assay. Viral yields after different addition time of isoquercetin (2 μM) to cell culture. MDCK cells were infected with influenza A/swine/OH/511445/2007 (Oh7 strain) at an MOI of 2. After 1 h of viral adsorption, 2 μM isoquercetin was added at the indicated times. At 12 h post-infection, virus titer was measured by $TCID_{50}$ assay.

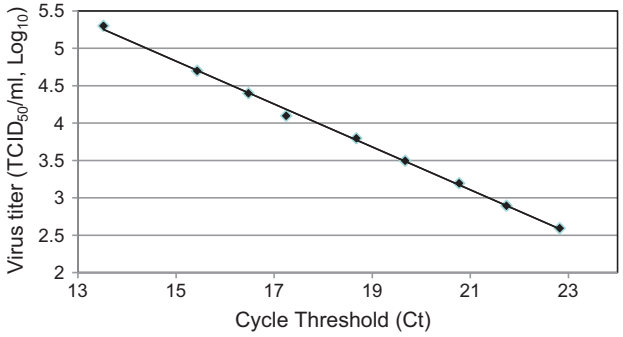

Fig. 5. Standard curve generated by plotting the Ct value versus Log_{10} titer of virus ($TCID_{50}$/ml). Nine-2 or 4-fold dilutions of stock virus (2×10^8 $TCID_{50}$/ml) were used to generate a standard curve, and a best-fit line was constructed with a correlation coefficient of 0.9982. Influenza virus quantity in clinical samples, expressed as log_{10} $TCID_{50}$/ml equivalents, is derived by plotting the Ct of a sample on the standard curve.

Table 5
Histopathology score of lungs from mice inoculated with PBS or virus with or without isoquercetin treatment (2 or 10 mg/kg/day).

Group	Score[a]
Virus	5
Virus + isoquercetin 2 mg/kg/day	NT[b]
Virus + isoquercetin 10 mg/kg/day	3.5
Isoquercetin 10 mg/kg/day	1
No treatment	0

Virus: mouse-adapted human influenza A virus [A/Puerto Rico/8/34, PR8 (H1N1)].
[a] Histopathology score ranges from 0 (normal) to 5 (severe inflammation).
[b] NT: not tested.

4. Discussion

Polyphenols have been studied for their antioxidant and anti-inflammatory effects, and recently it was shown that some of flavonoids exhibit antiviral actions against some viruses (Chen et al., 2006; Chiang et al., 2003; Choi et al., 2009a,b; Davis et al., 2008; Debiaggi et al., 1990; Docherty et al., 1999, 2006; Furuta et al., 2007; Heredia et al., 2000; Kaul et al., 1985; Mitrocotsa et al., 2000; Mori et al., 2008; Nakayama et al., 1993; Palamara et al., 2005; Song et al., 2005; Weber et al., 2003; Xiao et al., 2008). Among them, EGCG and resveratrol have been reported to have anti-influenza activity (Furuta et al., 2007; Mori et al., 2008; Palamara et al., 2005; Song et al., 2005; Xiao et al., 2008). In this study, we found that isoquercetin had lowest ED_{50} and higher in vitro therapeutic index among the polyphenols we tested including EGCG and resveratrol. To our knowledge, this is the first report that isoquercetin has antiviral effects against various influenza A and B viruses. Isoquercetin is a

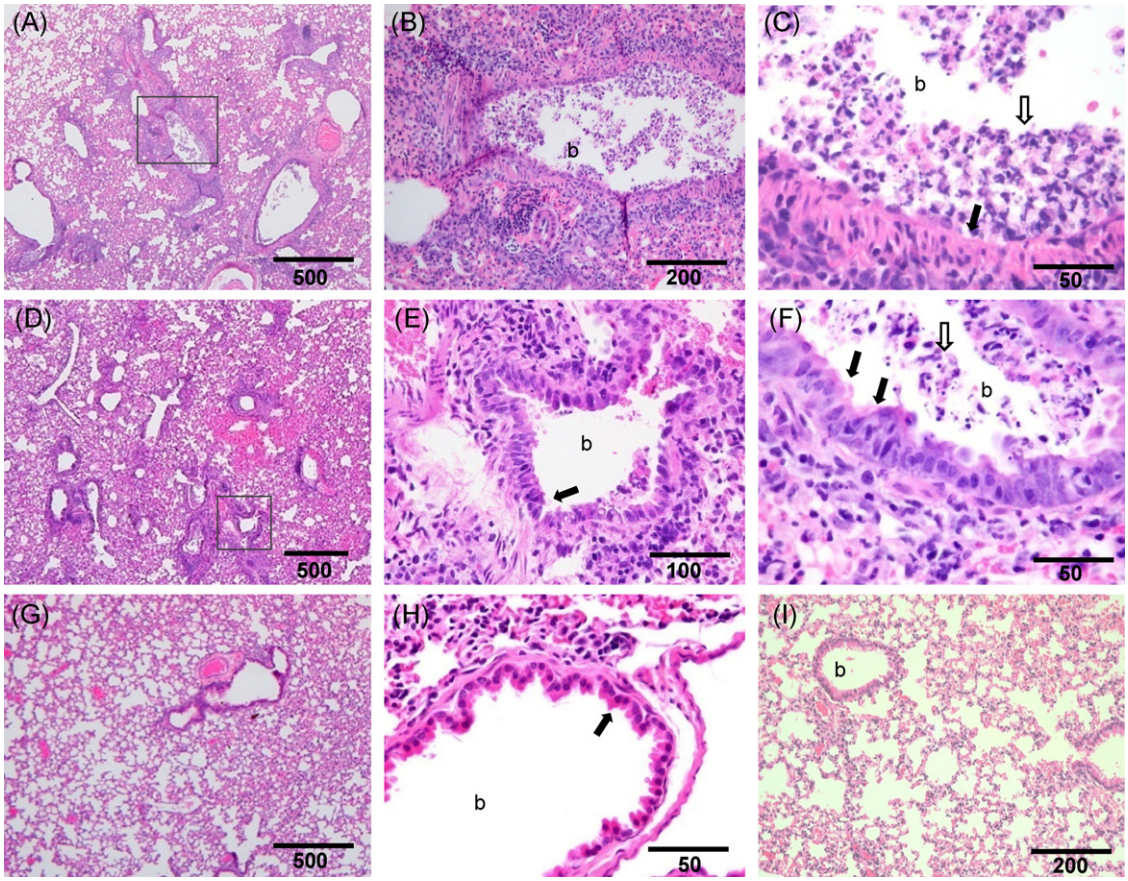

Fig. 6. Photomicrographs of lungs of BALB/C mice 6-day post-inoculation with PR8 virus (Hematoxylin and Eosin staining). Infected lung without treatment (Panels A–C); infected lung treated with isoquercetin (10 mg/kg/day) (Panels D–F); uninfected lung treated with isoquercetin (10 mg/kg/day) (Panels G and H); Uninfected lung without treatment (negative control) (Panel I). Panels A, B (magnified view of a box in Panel B) and C show marked infiltration of inflammatory cells in the alveolar walls and peribronchiolar and perivascular regions with loss of bronchiolar epithelial lining (solid arrow) and the presence of serocellular exudates (open arrow) in the lumen. Panels D, E (magnified view of a box in Panel D) and F shows alveolitis and bronchiolitis, however, with intact bronchiolar epithelium (solid arrows) even in the presence of moderate serocellular exudates in the lumen (open arrow). Panels G–I show intact columnar epithelium in the bronchiole (solid arrow). The bars represent sizes in microns. 'b' indicates airways.

glucose-bound derivative of quercetin, and is also reported to have anti-inflammatory and antioxidant activity (Chanh et al., 1986; Lee et al., 2008). However, there has been no previous report of antiviral activity of isoquercetin against influenza virus.

In addition to isoquercetin, quercetin also showed a moderate antiviral activity against influenza virus when tested in our laboratory. However, interestingly, other quercetin derivatives such as rutin (quercetin-3-O-rutinoside), quecertin-3-galactose, and quercitrin (quercetin-3-O-rhamnoside) showed minimal antiviral activity against influenza virus. Previously, Davis et al. (2008) showed that quercetin (at 12.5 mg/kg) administered orally to mice for 7 days prior to influenza virus challenge (0.04 HA) (but not after) offset stress-induced increase in morbidity, symptom severity and mortality. However, the effects of quercetin were attributed to antioxidant activity of quercetin in that study (Davis et al., 2008). We found that quercetin has moderate anti-influenza effects (ED_{50} of 48 μM) similar to resveratrol in vitro. Thus, the effects of quercetin in that study could also have been contributed by antiviral effects of quercetin. Resveratrol was shown to inhibit the replication of influenza A virus in vitro, and oral administration of resveratrol significantly improved survival and decreased lung viral titers in a mouse model (Palamara et al., 2005). The antiviral mechanism of resveratrol against influenza virus was suggested to be associated with the inhibition of cellular signaling pathway (PKC/MAPK pathway) leading to retention of viral ribonucleoprotein in nucleus, thereby reduction of virus replication (Palamara et al., 2005). There are also several reports that EGCG resulted in reduction of the replication influenza virus in vitro without detailed mechanisms (Furuta et al., 2007; Mori et al., 2008; Nakayama et al., 1993; Song et al., 2005; Xiao et al., 2008). We found that isoquercetin did not have significant direct antiviral effect, and incubation of cells with isoquercetin prior to viral infection followed by extensive washing significantly inhibited viral replication. The addition of isoquercetin to cells up to 4 h following viral infection reduced virus replication in a time-dependent manner. These results suggest that the antiviral mechanism of isoquercetin may involve early stages of viral replication.

Combination treatments have been studied by other researchers to increase efficacy and to overcome viral resistance. In mice infected with H5N1 viruses, combination treatment of amantadine and oseltamivir led to greater protection from lethal infection than monotherapy (Ilyushina et al., 2007; Smee et al., 2009). A combination of oseltamivir and rimantadine showed synergistic effects against H3N2 influenza virus in vitro (Govorkova et al., 2004). In our study, synergistic effects were seen by the double treatment of isoquercetin and amantadine at the tested concentrations with highest synergy at lower concentrations of each compound in cell culture.

It has been reported that drug resistance emerges only after three or five passages in the presence of amantadine in cell culture, and up to approximately one-third of patients may shed resistant viruses when treated with amantadine or rimantadine

(Hayden et al., 1991). In our study, the viruses passaged in the presence of amantadine (2 μM) were resistant to amantadine even at 100 μM at passage 3. With oseltamivir, emergence of resistance was slower than amantadine, but ED_{50} greatly increased at passage 10. Interestingly, the viruses passaged in the presence of isoquercetin maintained the same ED_{50} for the duration of passages (10). It is important to note that the double treatment of isoquercetin and oseltamivir or amantadine suppressed the emergence of resistant viruses to amantadine or oseltamivir in the cells infected with influenza viruses in vitro. The double treatment of isoquercetin and oseltamivir or amantadine gradually decreased virus titers to undetectable titer levels, indicating the double treatment completely inhibited the replication of viruses.

Since isoquercetin showed antiviral effects in vitro, a mouse study was performed to determine in vivo effects of isoquercetin against mouse-adapted influenza A virus. Influenza virus-infected mice that did not receive treatment showed high lung viral titers on 6-day post-infection. Isoquercetin administration at 10 mg/kg/day (i.p.) significantly reduced the levels of lung viral titers compared to those of untreated mice. The isoquercetin treatment at both dosages significantly reduced the levels of IFN-γ, iNOS and RANTES in the lungs, compared to those of the untreated group. Quercetin and its glucoside forms such as isoquercetin are reported to scavenge free radicals and interfere with NOS activity to play a protective role in ischemic tissue damages (Fraga et al., 1987; Halliwell, 1994; Lakhanpal and Rai, 2007; Shoskes, 1998; Shutenko and Pinard, 1990), and were associated with lower eosinophils in bronchoalveolar lavage fluid and lung parenchyma in a mouse model of asthma (Rogerio et al., 2007). In mice, iNOS is expressed by exudate macrophages and bronchial epithelial cells in lung tissues infected with influenza virus mediated by proinflammatory cytokines such as IFN-γ (Akaike and Maeda, 2000). In pneumonia induced by influenza virus or Sendai virus, nitric oxide (NO) production was greatly attenuated in IFN-γ-deficient mice (Akaike and Maeda, 2000). These results strongly support the suggestion that IFN-γ is a major cytokine inducing iNOS and NO overproduction in pathogenesis of virus infection (Akaike and Maeda, 2000). RANTES (CCL5), expressed by airway epithelium as a potent chemoattractant for monocytes, T cells, basophils and eosinophils, is another indicator for inflammation (Matsukura et al., 1998). It was shown previously that administration of L-NMMA, a relatively non-selective inhibitor of all nitric oxide synthase (NOS) isoforms, significantly increased survival rate in mice, indicating the pathogenic role of NO overproduction in influenza virus-induced pneumonia in mice (Akaike et al., 1996). However, importantly, NOS inhibition by L-NMMA did not significantly affect propagation of influenza virus in vivo in either lethal or sublethally infected mice, suggesting that reduction in NO overproduction itself did not affect viral replication. The viral titers in mice received isoquercetin at lower dose (2 mg/kg/day) did not decrease significantly compared to those in untreated mice. However, interestingly, the levels of IFN-γ, iNOS and RANTES in mice received lower dose of isoquercetin (2 mg/kg/day) were significantly reduced compared to those of untreated mice, and comparable to the group received higher dose (10 mg/kg/day) of isoquercetin. These findings suggest that higher dose of isoquercetin was required to significantly decrease both viral replication and lung inflammation.

In summary, our study provides a proof-of-principle demonstration that isoquercetin significantly reduced the virus replication and lung pathology in influenza A virus infection in vivo. In addition, suppression of emergence of resistant virus to amantadine or oseltamivir by isoquercetin co-treatment in vitro suggests the potential use of isoquercetin as a therapeutic agent for the treatment of influenza virus infection and for the suppression of resistance in combination therapy with existing drugs.

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