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Antiviral effect of strictinin on influenza virus replication

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ABSTRACT

Strictinin, which is a member of the ellagitanin family of hydrolyzable tannins, prevented replication of human, duck and swine influenza A viruses (IAVs) in vitro at non-toxic concentrations. The addition of strictinin at the same time as IAV inoculation to MDCK cells inhibited viral replication in a dose-dependent manner. Strictinin showed 50% inhibitory concentrations for IAVs from 0.09 ± 0.021 to 0.28 ± 0.037 μM (mean ± S.E.M.) by the focus-forming assay. Treatment of MDCK cells with strictinin before and after viral inoculation resulted in no significant antiviral activity. Further studies showed that strictinin inhibited IAV-induced hemifusion. However, strictinin exhibited no inhibitory effect against receptor binding, sialidase activity. Strictinin also showed an antiviral effect on influenza B virus and human parainfluenza virus type-1 in vitro. The results indicate that strictinin is a useful antiviral agent.

1. Introduction

In late March and early April of 2009, an outbreak of swine-origin H1N1 influenza A virus (IAV) infection was detected in Mexico, with subsequent cases observed in several other countries including the United States (reviewed by Cheong, 2009). Transmission of highly pathogenic avian H5N1 viruses from poultry to humans has raised fears of an impending influenza pandemic (Khurana et al., 2009). Presently, there are two classes of anti-influenza virus drugs licensed by the U.S. Food and Drug administration that are effective against IAVs, including H5N1 viruses. These drugs are M2 channel blockers (amantadine and rimantadine), and sialidase inhibitors (zanamivir and oseltamivir) (De Clercq, 2004; Deyde et al., 2009). All four drugs are effective upon prompt administration after infection or prophylactically, but concerns have been raised by the isolation of viable mutant viruses that are resistant to them (Gubareva et al., 2001; Carr et al., 2002; Hayden and Hay, 1992). Since 2005, a dramatic rise in the resistance of seasonal IAVs to admantanes led to changes in CDC recommendations for the use of admantanes in the control of influenza virus infections (CDC, 2006). The World Health Organization (WHO) is working to stockpile antiviral drugs to attempt to stop or delay pandemic influenza at its initial emergence (WHO, 2009). Additionally, recent data collected by the WHO demonstrate a sudden increase in oseltamivir-resistant influenza A/H1N1 viruses all over the world (Bouvier et al., 2008; Hurt et al., 2009). Therefore, there is a need to develop new antiviral agents for therapeutics.

Tea, one of the most popular beverages in the world, is normally produced from the leaves of two cultivated tea plants, *Camellia sinensis* (L.) O. Kuntze var. sinensis and *C. sinensis* var. assamica (Masters) Kitamura (Theaceae). On the basis of the processing procedures, it can be generally divided into green tea (nonfermented), oolong tea (semi-fermented), black tea (fully fermented by polyphenol oxidase), and Pu-er tea (post-fermented by microbes) (Gao et al., 2008). It is well known that green tea contains catechins, including epigallactocatechin 3-*O*-gallate (EGCG), epigallactocatechin, epicatechin 3-*O*-gallate, epicatechin, and gallactocatechin 3-*O*-gallate, as the major polyphenols, and that the catechins show multiple biological activities including IAV inhibition activity (Hara, 2001; Song et al., 2005). In the tea plant, hydrolyzable tannins (for example, strictinin and theogallin) are also present as minor polyphenols (Nonaka et al., 1983; Yagi et al., 2009). The major monomeric catechins extracted from green tea comprised about 10–20% of the product's dry weight, whereas strictinin extracted from green tea comprised about 0–1.0% of the products dry weight (Niino et al., 2005). Strictinin (Fig. 1) is a member of the ellagitanin family of hydrolyzable tannins (reviewed by Quideau and Feldman, 1996). Ellagitannins contain at least one axially chiral diphenic acid unit and have been reported to have important biological activities such as anti-tumor

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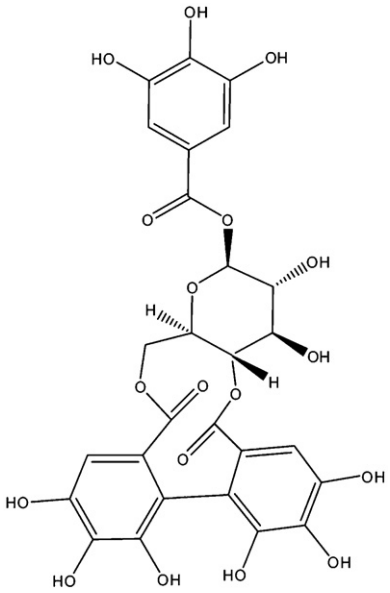

Fig. 1. Chemical structure of strictinin.

and antiviral actions on herpes simplex virus (HSV-1, HSV-2) and anti-topoisomerase activity (Kashiwada et al., 1992; Fukuchi et al., 1989; Bastow et al., 1993). Strictinin also shows antioxidative and antiallergic activities (Zhou et al., 2004; Tachibana et al., 2001). Moreover, strictinin is a promoter in the formation of the catechin–polysaccharide complex, which is a potential immunostimulator (Monobe et al., 2008). It has been reported that tannins of various chemical structures occurring in medicinal and food plants that are utilized worldwide showed several remarkable biological and pharmacological activities that are often specific to certain tannin structures and are significant for human health (reviewed by Okuda, 2005). Here, we demonstrated antiviral effects of strictinin on the replication of IAVs in vitro. We found that treatment with strictinin at the same time as IAV inoculation induced distinct reduction in IAV replication. We also showed that strictinin inhibited the replication of influenza B virus (IBV) and human parainfluenza virus type-1 (hPIV-1) in vitro.

2. Materials and methods

2.1. Compounds

Strictinin and EGCG were purchased from Nagara Science Co., Ltd., Japan. Fetuin, Amantadine, o-phenylenediamine (OPD), bovine serum albumin (BSA) and sialidase inhibitor{(4S,5R,6R)-5-acetamido-4-hydroxy-6-[(1R,2R)-1,2,3-trihydroxypropyl]-5,6-dihydro-4H-pyran-2-carboxylic acid} (DANA) were purchased from Sigma–Aldrich, Co., MI, USA. Hybridoma-SFM complete DPM (HSFM) was purchased from Invitrogen Corporation, CA, USA. The fluorogenic sialidase substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-Neu5Ac) and Nonidet P-40 (NP-40) were purchased from Nacalai Tesque Inc., Tokyo, Japan. All chemicals were of the highest purity available.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) and rhesus monkey kidney (LLC-MK2) cells were grown in minimum essential medium (MEM, Invitrogen Corporation, NY, USA) supplemented with 5% heat-inactivated (v/v) fetal bovine serum (Sigma, St. Louis) and 50 μg/ml gentamicin (Invitrogen Corporation, NY, USA). The human epithelial lung cell line A549 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corporation, NY, USA) supplemented with 10% (v/v) heat-inactivated FBS and gentamicin (50 μg/ml). A/PuertoRico/8/34 (H1N1), A/Memphis/1/71 (H3N2), A/Aichi/2/68 (H3N2), A/duck/HK/313/4/78 (H5N3), A/swine/Hokkaido/10/85 (H3N2), A/WSN/33 (H1N1) and B/Lee/40 were propagated in the allantoic cavities of 11-day-old chicken eggs for 48 h at 34.5 °C and purified as described previously (Suzuki et al., 1983). Pandemic 2009 strain A/Shizuoka/838/2009 (H1N1) was propagated in MDCK cells cultured with 50 ml of HSFM containing acetylated trypsin (2 μg/ml) at 34.5 °C until a 50% cytopathic effect was observed and then purified as described above. Human parainfluenza virus type-1 (hPIV-1) was isolated as previously described method (Suzuki et al., 2001). In brief, confluent monolayers of LLC-MK2 cells were infected with hPIV-1 strain (approximately 10 focus-forming unit; FFU/cell) in HSFM containing acetylated trypsin (1 μg/ml). Three days after infection, virions in the culture medium were collected. Hemagglutinin unit (HAU) of each virus was defined as the maximum dilution of virus that caused hemagglutination after 2 h on ice as described previously (Suzuki et al., 1983).

2.3. Antibodies

Rabbit anti-A/Memphis/1/71 (H3N2) antibodies, anti-B/Lee/40 antibodies, anti-hPIV-1 antibodies, Mouse anti-NP monoclonal antibody (MAb), and anti-HA MAbs (2E10 and IF8) were prepared as described previously (Suzuki et al., 2001a,b; Takahashi et al., 2008). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG + M antibodies, HRP-conjugated goat anti-mouse IgG antibodies (Jackson Immuno Research, West Grove, PA) and HRP-conjugated goat anti-rabbit IgG antibodies (ICN Pharmaceuticals Inc.) were purchased from Wako Inc., Japan.

2.4. Cytotoxicity test by MTT assay

Confluent MDCK cell monolayers in 24-well plastic plates (Falcon 353047, Becton Dickinson, NJ) were incubated with various concentrations of strictinin (0–75 μM) in HSFM and were observed microscopically for morphological changes after 72 h of incubation.

The effects of strictinin on the proliferation of MDCK, A549 and LLC-MK2 cells were determined in 96-well plastic microtiter plates (Falcon 353072, Becton Dickinson, NJ) by the tetrazolium-based colorimetric-MTT assay (Mosmann, 1983) using a cell proliferation kit 1 (MTT) (Cat. No. 11 465 007 001, Roche Diagnostics GmbH, Mannheim, Germany) by following the manufacturer's protocol. Briefly, approximately 10^4 cells/well were seeded and incubated with HSFM containing various concentrations of strictinin (0–75 μM) for 72 h before assay. The 50% cytotoxic concentration (CC_{50}) was calculated by 50% reduction of the optical density values (OD_{550}) with respect to control treated cells without inhibitors.

2.5. Focus-forming assay

Focus-forming assay was performed as described previously (Saha et al., 2009). The cells tested were fixed with ice-cold methanol for 30 s. The cells were then incubated with anti-nucleoprotein (NP) MAb diluted 1:4 in phosphate buffer saline, PBS (50 μl/well) for IAVs, rabbit anti-B/Lee/40 antibody diluted 1:300 in PBS (300 μl/well), or anti-hPIV-1 antibody diluted 1:300 in PBS (300 μl/well) for 60 min at room temperature. After washing with PBS, HRP-conjugated goat anti-moue IgG + M (Jackson Immuno Research, West Grove, PA) diluted 1:3000 in PBS (50 μl/well) or HRP-conjugated goat anti-rabbit IgG antibody diluted 1:3000 in PBS (300 μl/well) was incubated for 60 min at

room temperature. Virus-infected cells were detected by addition of an immunostaining reagent containing (50 μl/well) H_2O_2, *N*,*N*-diethyl-p-phenylenediamine dihydrochloride, and 4-chloro-1-naphtol (Conyers and Kidwell, 1991). All pictures were taken by using an OLYMPUS IX71 at a magnification of 12× and foci were counted using Adobe Photoshop CS2 software (Adobe Systems, CA). Number of foci was determined as focus-forming units (FFU). Standard error of the mean (S.E.M.) was calculated from results of three independent experiments.

2.6. Plaque forming assay

MDCK cells (0.5×0^5/well) were seeded in a 24-well plastic plate (Falcon 353047, Becton Dickinson, NJ). After washing with PBS, the cells were inoculated with log dilutions of HSFM containing IAV for 1 h at 37 °C. After washing with PBS, the cell monolayers were overlaid with HSFM containing acetylated trypsin and 0.8% agarose. The monolayers were incubated at 34 °C until plaques could be visualized as previously described (Takahashi et al., 2008).

2.7. Inhibition of IAV replication by strictinin

MDCK cells (1.5×10^4/well) were cultured in a 96-well microplate (Falcon 353072, Becton Dickinson, NJ) for 12 h at 37 °C before inoculation of IAV. After washing with HSFM, the cells were inoculated with each IAV strain suspension (0.01 FFU/cell) in HSFM containing various concentrations of strictinin (0–6 μM) for 1 h on ice. Then after washing with HSFM, the cells were incubated with HSFM containing various concentrations of strictinin (0–6 μM) for 12 h at 37 °C. Antigen-positive cells were detected with anti-NP MAb and counted by using Adobe Photoshop CS2 software as described in focus-forming assay. EGCG and amantadine hydrochloride were used as a positive control instead of strictinin. S.E.M. was calculated from results of three independent experiments.

To investigate anti-virus effects of strictinin at different stages of IAV replication, the effects of strictinin on IAV infection were determined by three protocols (Fig. 3) as previously described (Saha et al., 2009). In the first protocol (Protocol 1), MDCK cells (1.5×10^4/well) in 96-well microplates were pre-incubated with HSFM containing various concentrations of strictinin (0–6 μM) for 12 h at 37 °C before inoculation of IAV to demonstrate the effect of strictinin on cellular factors responsible for viral infection. After washing with HSFM, the cells were inoculated with A/Memphis/1/71 (H3N2) virus suspension (0.01 FFU/cell or 2 FFU/cell) in HSFM for 1 h at 37 °C. After washing with HSFM, the cells were incubated with HSFM for 12 h at 37 °C. In the second protocol (Protocol 2), virus suspension (0.01 FFU/cell or 2 FFU/cell) was mixed with various concentrations of strictinin (0–6 μM). MDCK cells were inoculated with the virus suspension containing strictinin in HSFM for 1 h at 37 °C to demonstrate the direct effect of strictinin against IAV. After washing with HSFM, the cells were incubated with HSFM for 12 h or 24 h at 37 °C. In the third protocol (Protocol 3), MDCK cells were inoculated with the virus suspension (0.01 FFU/cell or 2 FFU/cell). After washing with HSFM, the cells were incubated with HSFM containing various concentrations of strictinin (0–6 μM) for 12 h or 24 h at 37 °C.

Antigen-positive cells after inoculation with the virus suspensions at low multiplicity of infection (moi) according to Protocols 1, 2 and 3 were detected with anti-NP MAb and counted by using Adobe Photoshop CS2 software. The progeny virus titer of the culture medium at 24 h after inoculation with high moi according to Protocols 2 and 3 was determined by a focus-forming assay or plaque forming assay.

2.8. Inhibition of IBV replication by strictinin

MDCK cells (1.0×10^5/well) were cultured in MEM supplemented with 5% (v/v) heat-inactivated FBS and gentamicin (50 μg/ml) in a 12-well plate (Falcon 353043 MULTIWELL™ 12-well, Becton Dickinson, NJ). After washing with PBS, the cells inoculated with influenza virus B/Lee/40 suspension (0.003 FFU/cell) in HSFM containing different concentrations of strictinin (0–8 μM) for 1 h at 34.5 °C. After washing with PBS, the cells were incubated with the mixture of HSFM containing the same concentrations of strictinin (0–8 μM), 1.2% avicel and 2 μg/ml of acetylated trypsin for 48 h at 34.5 °C. IBV-infected cells were determined by the focus-forming assay using rabbit anti-B/Lee/40 antibody diluted 1:300 in PBS (300 μl/well) and HRP-conjugated goat anti-rabbit IgG antibody diluted 1:3000 in PBS (300 μl/well) as described above. S.E.M. was calculated from results of three independent experiments. All pictures were scanned and prepared by Adobe Photoshop CS2 software (Adobe Systems, CA).

2.9. Inhibition of hPIV-1 replication by strictinin

LLC-MK2 cells (1×10^5/well) were cultured in MEM containing 5% (v/v) heat-inactivated FBS and gentamicin (50 μg/ml) in 12-well plates. After washing with PBS, the cells were inoculated with hPIV-1 virus suspension (0.003 FFU/cell) in HSFM containing different concentrations of strictinin (0–20 μM) for 1 h at room temperature. After washing with PBS, the cells were incubated with the mixture of HSFM containing the same concentrations of strictinin (0–20 μM), 1.2% avicel and 2 μg/ml of acetylated trypsin for 48 h at 37 °C. hPIV-1-infected cells were determined by the focus-forming assay using rabbit anti-hPIV-1 antibody diluted 1:300 in PBS (300 μl/well) and HRP-conjugated goat anti-rabbit IgG antibody diluted 1:3000 in PBS (300 μl/well) as described above. S.E.M. was calculated from results of three independent experiments. All pictures were scanned and prepared by Adobe Photoshop CS2 software (Adobe Systems, CA).

2.10. Sialidase inhibition assay

Sialidase activity was determined by a modified fluorometric assay previously described (Suzuki et al., 1992). Two microliters of influenza virus A/Memphis/1/71 (H3N2) suspension (2^8 HAU) in 10 mM acetate buffer (pH 6.0) was incubated with 1 μl of various concentrations (0–430 μM) of strictinin solution for 60 min at 4 °C in each well of a 96-well microtiter plate (black, flat-bottom, BD Falcon, USA). The enzymatic reaction was initiated by addition of 1 μl of a substrate, 4-MU-Neu5Ac. After incubation for 30 min at 37 °C, the reaction was stopped by addition of 100 μl of 100 mM carbonate buffer (pH 10.7). The fluorescent intensity resulting from substrate cleavage by the viral sialidase activity was measured at 355 nm (excitation) and 460 nm (emission) using a multilabel counter Wallac 1420 ARVOsx (PerkinElmer, MA). DANA (0–330 μM) was used as a positive control. S.E.M. was calculated from results of three independent experiments.

2.11. Binding inhibition assay

Fifty microliters of PBS containing fetuin (5 mg/ml) was added to each well of microtiter plates (Corning Polystyrene Universal-BIND Microplate, USA). The plates were incubated at 4 °C in a dark condition for 12 h and then irradiated under UV light at 254 nm for 5 min. After washing with PBS containing 0.01% Tween-20 (T-PBS), the plates was blocked with 0.3 ml of T-PBS containing 0.25% lipid-free BSA at 4 °C for 12 h as described previously (Suzuki et al., 2001a,b). The plates were washed with T-PBS three times. An equal amount of influenza virus A/Memphis/1/71 (H3N2) suspen-

sion (2^8 HAU) and strictinin (0–4.1 mM) solution in PBS was mixed on ice. Fifty microliters of each mixture was added to the wells and incubated on ice for 5 h. After washing with ice-cold T-PBS five times, 50 μl of anti-A/Memphis/1/71 (H3N2) antibody diluted 1:300 in T-PBS was added to each well. After incubation for 2 h on ice, the plates were washed three times with ice-cold T-PBS and then incubated with HRP-conjugated goat anti-rabbit IgG antibody diluted 1:3000 in T-PBS (50 μl/well) on ice for 2 h. The plates were washed with ice-cold T-PBS four times and then incubated with 100 μl of substrate solution containing 4 mg of OPD and 0.01% H_2O_2 in 100 mM phosphate–citric acid buffer, pH 5.6, for 10 min at room temperature. The reaction was stopped with 100 μl of 1N H_2SO_4 and the absorbance was determined at 492 nm, with a reference wavelength of 630 nm. Fetuin (0–3.6 mM) solution in PBS was used as a positive control.

2.12. Labeling of erythrocytes with calcein-AM

Guinea pig erythrocytes (GRBCs) were labeled with calcein-acetoxymethyl ester (AM) (AnaSpec, Inc., San Jose, CA) by using a modification of a method described previously (Li et al., 2008). One milliliter of freshly prepared GRBCs (10% in PBS) was mixed with 10 μl of calcein-AM (1 mg/ml) in dimethyl sulfoxide and incubated for 2 h at 37 °C by gentle shaking at intervals of 30 min. After washing with PBS three times to remove unbound calcein-AM, calcein-labeled GRBCs (0.2%) were suspended in HSFM.

2.13. Dye transfer assay

COS-7 cells (1×10^5/well) were cultured in 24-well plastic plate (Falcon 353047, Becton Dickinson, NJ). The cells were inoculated with A/WSN/33 (H1N1) (MOI. 2) and incubated 12 h at 37 °C. After washing with PBS three times, the cells were pretreated with HSFM containing acetylated trypsin (2 μg/ml) for 3 h at 37 °C. The cells were incubated with various concentrations of strictinin (0, 1 and 10 μM) for 1 h at 37 °C. After washing with PBS three times, the cells were incubated with calcein-labeled GRBCs for 1 h at 4 °C for hemadsorption. After washing with PBS three times to remove unbound calcein-labeled GRBCs, the cells were incubated with acidic HSFM (pH 5.5) for 30 min at 37 °C. The transference of calcein from GRBCs to IAV-infected cells was observed under a fluorescence microscope (200×) and photographed. Six fields were chosen randomly from each well and their mean fluorescence intensity values were calculated by ImageJ 1.40g software (National Institute of Health, USA). As controls, IAV-infected COS-7 cells were incubated with calcein-labeled GRBCs without acetylated trypsin treatment. Additionally, GRBCs-adsorbed cells were incubated with HSFM (pH 7.0) instead of acidic HSFM (pH 5.0).

3. Results

3.1. Cytotoxicity of strictinin

Confluent MDCK cell monolayers treated for 72 h with strictinin at concentrations of 0–1 μM did not show any visible changes in cell morphology or cell density, whereas 5 μM of strictinin caused microscopically detectable alterations. To evaluate the effect of strictinin on proliferating cells, MDCK, A549 and LLC-MK2 cells were grown in HSFM containing strictinin (0–75 μM). After incubation for 72 h, cell viability was determined by the MTT assay. Strictinin at concentrations of 1 μM or less did not significantly reduce the OD_{550} values for strictinin-treated MDCK cells compared to the control. The CC_{50} of strictinin on MDCK cells was 5.1 ± 0.31 μM. The effect of strictinin on proliferating cells of A549 and LLC-MK2 cells was also investigated and about 60% of both

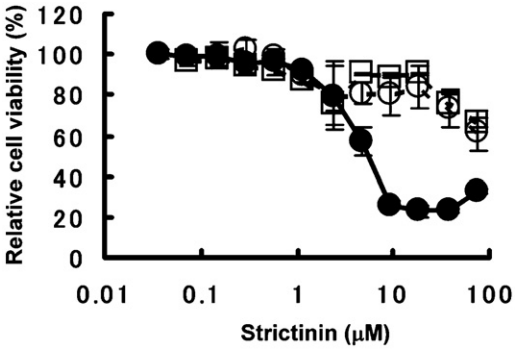

Fig. 2. Cellular toxicity of strictinin. The cytotoxic effects of strictinin on MDCK (closed circle), A549 (open circle) and LLC-MK2 (open rectangle) cell growth were examined. The cells were seeded with various concentrations of strictinin (0–75 μM), after incubation for 72 h at 37 °C cell viability was determined by the MTT assay as described in Section 2. The optical densities (OD_{550}) of compound-treated cells are expressed as a percentage of viable cells. Bar indicates S.E.M. of three independent experiments.

A549 and LLC-MK2 cells were viable in presence of 75 μM of strictinin (Fig. 2).

3.2. Inhibitory effects of strictinin on different stages of viral replication

We examined the anti-viral effect of strictinin in three different protocols as shown in Fig. 3. Treatment with strictinin (0–6 μM) at the same time as inoculation of influenza virus A/Memphis/1/71 (H3N2) to MDCK cells resulted in a clear decrease in the number of IAV-infected cells (Fig. 4A) and in progeny virus production (Fig. 4B) in comparison with strictinin treatment before and after viral inoculation (Fig. 4A and B). Strictinin also showed similar effects on IAV infection in A549 cells under the same conditions (Fig. 4C and D). The progeny virus production after 24 h in Protocols 2 and 3 of strictinin on A/Memphis/1/71 (H3N2) (MOI 2) was also investigated by a plaque assay. There was a clear decrease in the progeny viral titer when strictinin was added at the same time as viral infection and no inhibition was found when strictinin was added after viral infection (data not shown).

The effect of strictinin treatment at the same time as duck H5N3 and swine-origin H1N1 pandemic IAVs inoculation was evaluated in a dose-dependent manner (Fig. 5). Strictinin strongly inhibited viral replication in comparison with the effect of EGCG. The 50% inhibitory concentration (IC_{50}) values of strictinin treatment against different subtypes of IAV were determined from their dose–response curves. Strictinin inhibited infection of all IAVs tested more strongly than did EGCG and amantadine hydrochloride. Selectivity index (SI) was calculated from the ratio of CC_{50}/IC_{50} (Table 1). SI values of strictinin against IAVs were from 18 to 56.

3.3. Effects of strictinin on receptor binding and sialidase activities of IAV

The viral spike glycoprotein hemagglutinin (HA) binds to sialic acid on the surface of host cells and mediates membrane fusion for the viral uncoating process (Wiley and Skehel, 1987). We examined the effect of strictinin on IAV binding by a competitive inhibition assay with fetuin. Strictinin did not show any binding inhibition activity for A/Memphis/1/71 (H3N2) in a dose range of 0–4.1 mM (Fig. 6).

The viral spike glycoprotein neuraminidase destroys sialic acid-containing receptors on the surface of infected cells and on progeny virions (reviewed by Colman, 1999). Sialidase activity of NA is important for the initiation step of IAV infection and budding of IAV

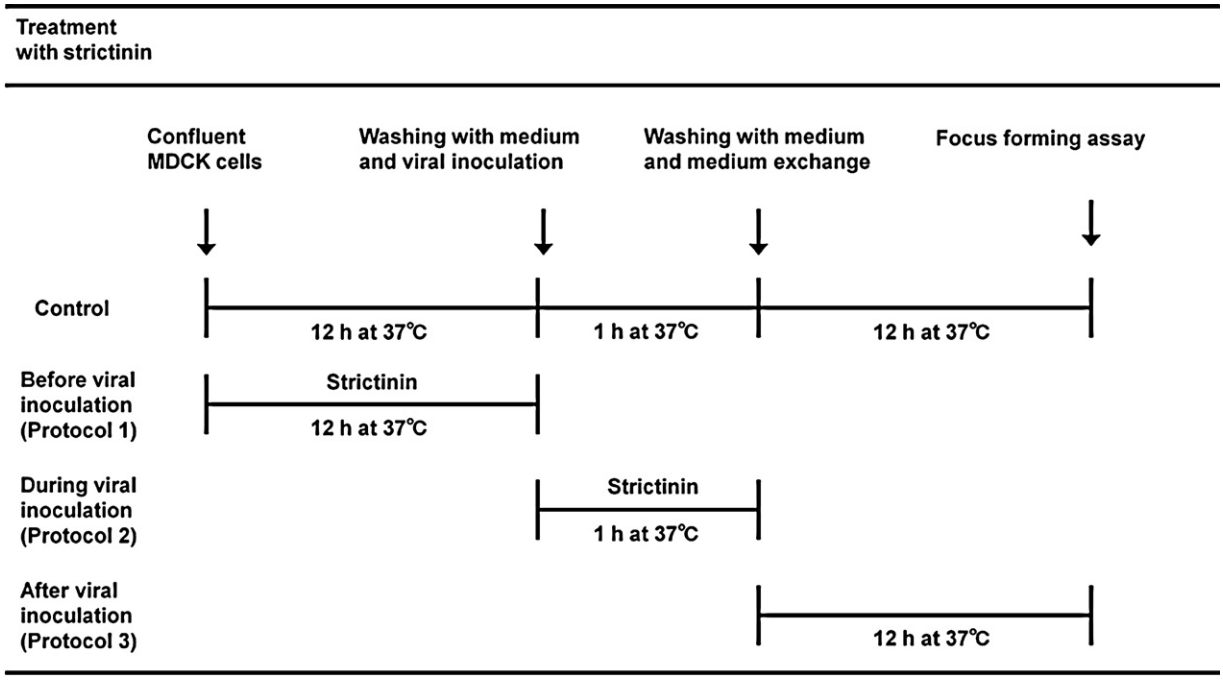

Fig. 3. Protocols of experiments to determine the effects of strictinin treatment on IAV infection. MDCK or A549 cells (1×10^4/well) and A/Memphis/1/71 (H3N2) strain (0.01 FFU/cell) for Protocols 1 and 2 and 3 were used in all protocols. The cells were cultured with HSFM containing various amounts of strictinin (0–6 μM) for 12 h at 37 °C and washed before viral inoculation to evaluate the effect of strictinin on the cells (Protocol 1). Viral suspension was inoculated with various amounts of strictinin (0–6 μM) during infection to evaluate the effect of strictinin in the entry stage of IAV infection (Protocol 2). IAV-infected cells were incubated with HSFM containing various amounts of strictinin (0–6 μM) for 12 h at 37 °C to evaluate the effect of strictinin in the stage after virus entry (Protocol 3).

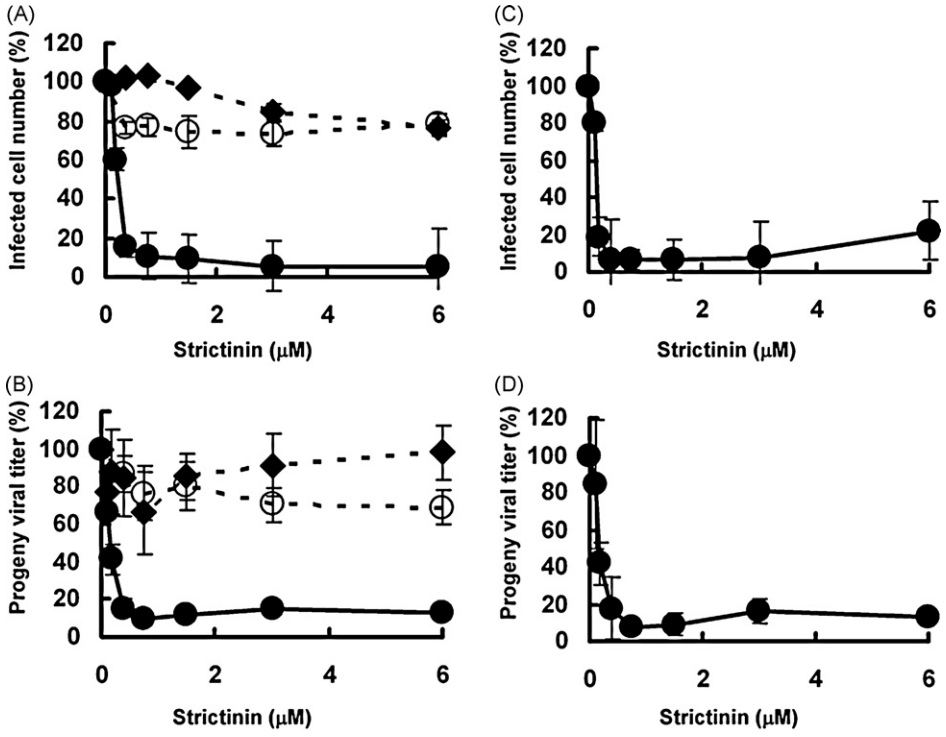

Fig. 4. Inhibitory effects of strictinin on IAV infection and progeny virus production. MDCK cells (1×10^4/well) were treated with various amounts of strictinin (0–6 μM) before, at the same time as, and after inoculation with A/Memphis/1/71 (H3N2) virus (0.01 FFU/cell or 2 FFU/cell) according to Protocols 1 (closed diamond), 2 (closed circle) and 3 (open circle). After incubation for 12 h at 37 °C, virus-infected cells under the condition of low multiplicity of infection (A) were detected with anti-NP MAb and counted by using Adobe Photoshop CS2 software as described Section 2, and the progeny virus titer of the culture medium under the condition of high multiplicity of infection (B) was determined by a focus-forming assay. A549 cells (1×10^4/well) were treated with various amounts of strictinin at the same time as viral inoculation. After incubation for 12 h at 37 °C, the number of virus-infected cells (C) and the progeny virus titer of the culture medium (D) were determined by a focus-forming assay. Initial infectivity and progeny viral titer were expressed as a percentage relative to the total number of viral antigen-positive cells per area of a well without strictinin treatment under the same condition. S.E.M. was calculated from results of three independent experiments.

Table 1
Anti-viral activity of strictinin, EGCG, and amantadine against IAV strains.

Strain	Strictinin		EGCG		Amantadine	
	IC_{50}[a] (μM)	Si[b]	IC_{50}[a] (μM)	Si[b]	IC_{50} (μM)	Si[b]
A/Memphis/1/71 (H3N2)	0.17 ± 0.024	30	0.52 ± 0.039	15	3.18 ± 0.095	N.D.[c]
A/Aichi/2/68 (H3N2)	0.19 ± 0.042	26.8	0.35 ± 0.033	22.3	0.62 ± 0.107	N.D.[c]
A/duck/HK/313/4/78 (H5N3)	0.22 ± 0.017	23.2	0.55 ± 0.056	14.2	0.59 ± 0.089	N.D.[c]
A/Shizuoka/838/2009 (Pandemic H1N1)	0.09 ± 0.021	56.7	0.12 ± 0.019	65	>50	N.D.[c]
A/PR/8/34 (H1N1)	0.28 ± 0.037	18.2	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]
A/swine/Hokkaido/10/85 (H3N2)	0.24 ± 0.022	21.3	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]

MDCK cells (1×10^4/well) were inoculated with HSFM containing each IAV strain (0.01 FFU/cell) and various amounts of strictinin (0–0.6 μM), EGCG (0–3.6 μM) and amantadine hydrochloride (0–50 μM) for 1 h on ice. After washing with HSFM, the cells were incubated with HSFM containing the same concentrations of strictinin, EGCG and amantadine hydrochloride for 12 h at 37 °C. Virus-infected cells were determined by a focus-forming assay and the concentration causing 50% inhibition of antigen-positive cell number ([a]IC_{50}) was calculated from the dose–response curve. IC_{50} is presented as mean ± S.E.M. ($n = 3$). The 50% cytotoxic concentration (CC_{50}) was determined by MTT assay. CC_{50} values of strictinin was 5.1. Selectivity index ([b]SI) is presented as the ratio of CC_{50} to IC_{50}. [c]N.D. means not determined.

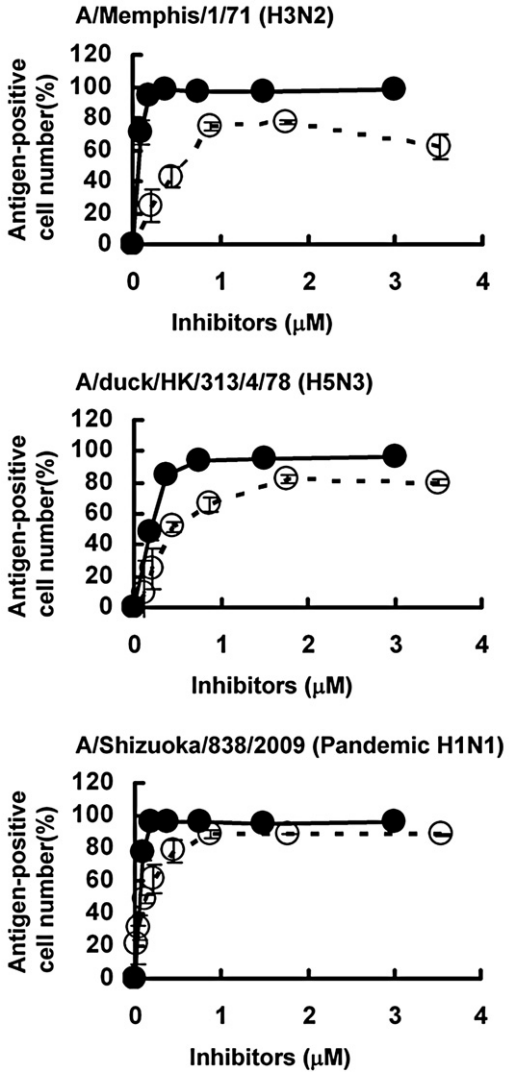

Fig. 5. Effects of strictinin on infection of duck H5N3 and swine-origin H1N1 pandemic IAVs. MDCK cells (1×10^4/well) were inoculated with HSFM containing each IAV strain (0.01 FFU/cell) and various amounts of strictinin (0–3 μM) or EGCG (0–3.6 μM) for 1 h on ice. After washing with HSFM, the cells were again incubated with HSFM containing the same amount of strictinin or EGCG for 12 h at 37 °C. Inhibition of IAV infection by strictinin (closed circle) and EGCG (open circle) was expressed as a percentage relative to the total number of viral antigen-positive cells per area of a well. S.E.M. was calculated from results of three independent experiments.

particles (Matrosovich et al., 2004; Suzuki et al., 2005). Therefore, we examined the inhibitory effect of strictinin on viral sialidase activity by a fluorometric assay with 4-MU-Neu5Ac as previously described (Suzuki et al., 1992). Strictinin had no inhibitory effect on the sialidase activity of A/Memphis/1/71 (H3N2) (Fig. 7).

3.4. Effects of strictinin on IAV-induced hemifusion

To determine whether strictinin inhibits IAV-induced hemifusion, we measured the transference of calcein-AM from GRBCs to COS-7 cells infected with A/WSN/33 (H1N1). Calcein produced by

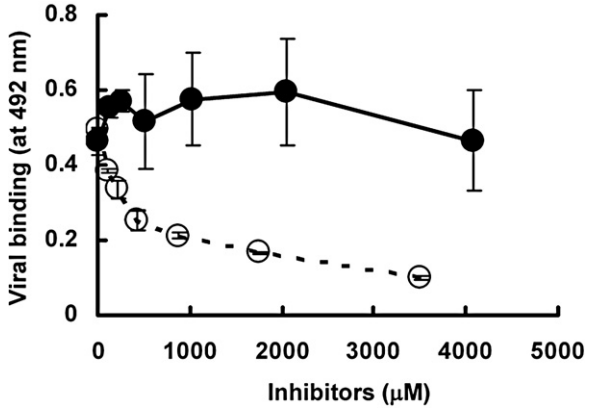

Fig. 6. Effect of strictinin on receptor binding of IAV. Inhibitory effect of strictinin (closed circle) on receptor binding of A/Memphis/1/71 (H3N2) was determined by a binding inhibition assay as described in Section 2. Fetuin (open circle) was used as a positive control. Bars indicate S.E.M. calculated from results of three independent experiments.

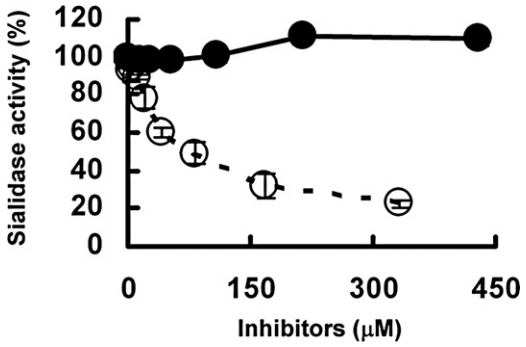

Fig. 7. Effect of strictinin on IAV sialidase activity. A/Memphis/1/71 (H3N2) was incubated with strictinin solution (closed circle) serially diluted for 60 min at 4 °C. Sialidase activities were assayed by the fluorometric assay as described in Section 2. As a control, DANA (open circle) was used instead of strictinin. The experiments were carried out in triplicate. Bars indicate S.E.M. of three independent experiments.

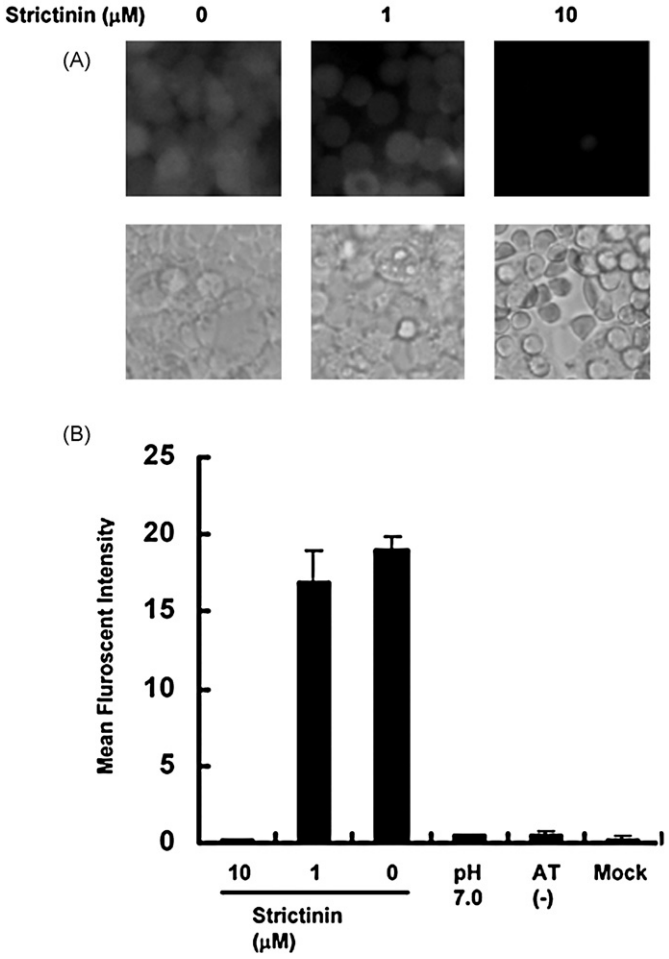

Fig. 8. Effect of strictinin on IAV-induced hemifusion. COS-7 cells (1×10^5/well) were inoculated with A/WSN/33 (2 PFU/cell) and incubated 12 h at 37 °C. After pretreatment with acetylated trypsin (2 μg/ml) and strictinin (0, 1 and 10 μM), the cells were incubated with calcein-AM-labeled GRBCs for 1 h at 4 °C for hemadsorption as described in Section 2. After incubation with acidic HSFM (pH 5.5) for 30 min at 37 °C, the transference of calcein from GRBCs to IAV-infected cells was observed under a fluorescence microscope (A) and mean fluorescence intensity values of each well were determined by ImageJ 1.40 g software (B). pH 7.0; incubation with HSFM instead of acidic HSFM, AT (−); without acetylated trypsin treatment, Mock; mock-inoculation. The experiments were carried out in triplicate. Bars indicate S.E.M. of three independent experiments.

cytoplasmic esterases in COS-7 cells was observed under a fluorescent microscope (200×). Dye transfer assay showed that strictinin inhibited IAV-induced hemifusion (Fig. 8).

3.5. Effects of strictinin on IBV and hPIV-1replication

To investigate the overall effects of strictinin on IBV replication, MDCK cells were inoculated with a mixture of influenza virus B/Lee/40 and strictinin at various concentrations (0–4 μM). After washing with PBS, the cells were incubated with HSFM containing the same concentrations of strictinin. Treatment with strictinin at micromolar concentrations resulted in an obvious decrease in the number of IBV-infected cells (Fig. 9).

We also examined the inhibitory effects of strictinin on hPIV-1 replication.

LLC-MK2 cells were inoculated with a mixture of hPIV-1 and strictinin at various concentrations (0–20 μM). After washing with PBS, the cells were incubated with HSFM containing the same concentrations of strictinin. Strictinin inhibited hPIV-1 replication in a dose-dependent manner (Fig. 10).

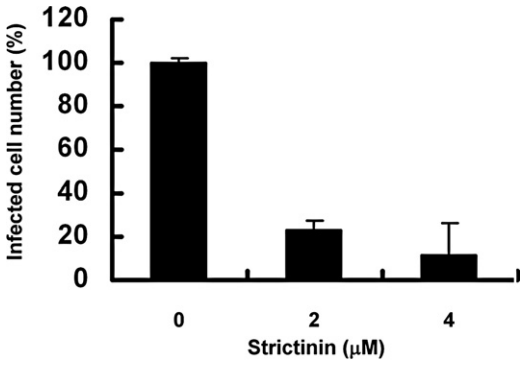

Fig. 9. Effect of strictinin on IBV infection. MDCK cells (1×10^5/well) were inoculated with a mixture of B/Lee/40 (0.003 FFU/cell) and various amounts of strictinin (0–4 μM) as described in Section 2. The viral infectivity was determined by a focus assay and expressed as a percentage relative to the total number of viral antigen-positive cells in the absence of strictinin. Bars indicate S.E.M. of three independent experiments.

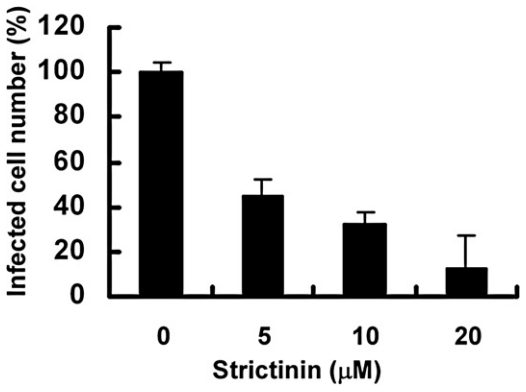

Fig. 10. Effect of strictinin on hPIV-1 infection. LLC-MK2 cells (1×10^5/well) were inoculated with a mixture of hPIV-1 virus (0.003 FFU/cell) and various amounts of strictinin (0–20 μM) as described in Section 2. The viral infectivity was determined by a focus assay and expressed as a percentage relative to the total number of viral antigen-positive cells in the absence of strictinin. Bars indicate S.E.M. of three independent experiments.

4. Discussion

We showed here that strictinin strongly inhibited IAV replication. IAV inoculation with strictinin resulted in a clear decrease in the number of IAV-infected cells for all viruses tested. However, strictinin treatment of MDCK cells before and after IAV inoculation had little effect on viral replication. The results suggested that strictinin directly reacted with the viral particles and inhibited viral entry in the initial stage. MDCK cells showed sensitivity different from that of other cells lines to the cytotoxic effect of strictinin. The canine kidney cell line MDCK and other cell lines are different in origin. The cytotoxic effect of strictinin may be involved in their biological properties. Tea polyphenols and green tea catechins are known to act directly on the influenza virus envelope (Nakayama et al., 1993; Song et al., 2005). In this study, strictinin also showed a virucidal effect on the enveloped viruses including IAV. However, tea polyphenolic compounds such as EGCG have multiple inhibitory effects at several stages of the viral replication cycle including receptor binding, viral RNA synthesis and sialidase activity (Song et al., 2005). Polyphenols act principally by binding to the virus and/or the protein of the host cell membrane and thus arrest absorption of the virus (reviewed by Haslam, 1996). Tannins and related compounds are known to inhibit binding of herpes simplex virus to African green monkey kidney cells and human adenocarcinoma cells and also inhibit HIV reverse tran-

scriptase by noncompetitive mechanism (Fukuchi et al., 1989; Ng et al., 1997).To investigate the effect of strictinin on the initial stage of the viral replication cycle, we examined the effects of strictinin on receptor binding and sialidase activities of IAV by a competitive inhibition assay with fetuin and by a fluorometric assay with 4-MU-Neu5Ac, respectively. The virucidal effect of strictinin differed from those of EGCG and tannins in that strictinin showed no inhibitory effect on viral binding and sialidase activity. Interestingly, strictinin inhibited IAV-induced hemifusion. Strictinin also inhibited the replication of not only IBV but also hPIV-1. Our present result indicates that strictinin was inferred to act on the entry stage of the IAV through its effects on viral fusion activities. Influenza A and B viruses release viral ribonucleoprotein complexes into the cytoplasm after HA-mediated fusion under a low pH condition of endocytic trafficking (Wiley and Skehel, 1987), while hPIV-1 fuses the viral envelope with the surface membranes of host cells and releases viral ribonucleoprotein complexes into the cytoplasm (Moscona, 2005). Amantandine has been shown to inhibit IAV uncoating for entry of vRNP into the nucleus in the initial stage of viral infection (Bui et al., 2000; Kato and Eggers, 1969; Martin and Helenius, 1991). However amantadine has no effect on replication of IBV at micromolar concentrations because the function of M2 is replaced by a different protein in IBV (reviewed by Lamb and Pinto, 1997). Our results therefore suggest that strictinin has a different target of the entry pathway from amantadine and that strictinin represents a novel class of entry inhibitor. Recent advances in the field of viral entry have lead to the development of antiviral agents that target several discrete steps in the viral entry process, a number of which are being tested in clinical trials (Moore and Doms, 2003). Therefore, strictinin could provide a much needed addition to the antiviral agents for combating the wide range of influenza viruses in the early stage of infection.

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