Antiviral activity and possible mechanisms of action of pentagalloylglucose (PGG) against influenza A virus

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Abstract

Influenza A virus (IAV) infection is a major public health threat leading to significant morbidity and mortality. The emergence of drug-resistant virus strains highlights urgent needs to develop novel antiviral drugs with alternative modes of action. Pentagalloyl glucose (PGG), a natural occuring polyphenolic compound, possesses broad spectrum of biological activities. In this study, we found PGG has anti-influenza virus activity and investigated its possible mechanism(s) of action *in vitro*. Both pre-incubation of virus prior to infection and post-exposure of infected cells with PGG significantly inhibited virus yields. Influenza virus-induced hemagglutination of chicken red blood cells was inhibited by PGG treatment, suggesting PGG can inhibit IAV infection by interacting with viral hemagglutinin. PGG did not affect viral protein synthesis or nuclear transport of viral nucleoprotein (NP), but largely reduced plasma membrane accumulation of NP protein at the late stage of replication cycle. Furthermore, PGG significantly reduced virus budding and progeny virus release from infected cells. Altogether, this study for the first time revealed that PGG can inhibit IAV replication with dual mode of action and offers new insights into its underlying mechanisms of antiviral action.

Keywords:

Influenza A virus; PGG; antiviral activity; mechanisms of action

1. Introduction

Influenza A virus (IAV) is a segmented, negative sense, single-stranded RNA virus belonging to the *Orthomyxoviridae* family[7]. It causes influenza, which is an acute, highly transmissible respiratory infectious disease in humans and animals. Annual seasonal epidemics and occasional pandemics of influenza result in significant morbidity and mortality in both humans and animals worldwide. Furthermore, the emergence of the highly pathogenic H5N1 avian influenza virus, which was associated with a mortality rate in excess of 60% in infected individuals [35], as well as the 2009 flu pandemic, a global outbreak of a swine-origin new strain of H1N1 influenza virus, have raised significant public health concerns for emergence of a potential novel highly pathogenic pandemic influenza [5, 8, 17]. Vaccination is one of the most effective means of prophylactic antiviral therapy, while antiviral medications constitute the first line of treatment following infection. Two classes of antiviral drugs including M2 channel blockers and neuraminidase inhibitors have proven to be clinically effective against influenza. However, due to the high mutation rate of these viruses, the emergence of drug-resistant viral strains against both classes of drugs has been reported [22, 36]. This highlights the urgent need for discovering novel antiviral drugs with alternative modes of action. Recently, evidence is accumulating to show that inhibition of intracellular signaling cascades required for virus replication is a novel alternative approach for anti-influenza therapy. The advantage of this strategy is that it can avoid the emergence of drug-resistant virus strains due to the fact that the target of the drug is a host factor(s) which is not affected by virus mutation [19-21].

Since many traditional medicinal plants have been reported to have strong antiviral activity [6, 30, 37], they offer a rich source for discovering novel antiviral compounds. In order to explore novel active compounds against IAV, we screened a number of natural compounds purified from different Chinese medicinal plants. 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG), a naturally occurring polyphenolic compound abundant in several medicinal plants, was found to exhibit anti-influenza virus activity at non-cytotoxic concentrations. A number of *in vitro* and *in vivo* studies have previously shown that PGG exhibits a wide range of biological activities [41], including anti-inflammatory [16], antioxidant[29], anti-angiogenic [25], antitumor [14], antibacterial activity [40], and a broad range of antiviral activity against respiratory syncytial virus (RSV) [39], Hepatitis B virus (HBV) [18] and Herpes simplex virus (HSV) [27-28, 31]. Moreover, it has been shown to have an inhibitory effect on viral enzymes such as integrase and reverse transcriptase of human immunodeficiency virus (HIV-1) [1], and NS3 protease of Hepatitis C Virus (HCV) [9]. Although the underlying mechanisms of its antiviral action remains to be fully elucidated, the wide spectrum of its antiviral activity against different viruses suggests that PGG may target common critical steps in virus-cell interaction rather than a specific viral pathogen.

In the present study, we investigated the antiviral activity and possible mechanism(s) of action of PGG against IAV *in vitro.*

2. Materials and methods

2.1. Compound

1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG) (chemical structure is shown in Fig. 1a) was isolated from the branches and leaves of *Phyllanthus emblica Linn*, and purified as described previously [42]. The purity levels achieved were over 98%. PGG was dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium for the following experiments.

2.2. Cells, viruses and virus infections

Mardin-Darby canine kidney (MDCK) cells and human alveolar epithelial cell line A549 cells were cultured in minimum essential medium (MEM, Invitrogen) and Dulbecco's modified Eagle's medium (DMEM, Invitrogen), respectively, supplemented with 10% (v/v) fetal bovine serum (FBS, Cell Culture Bioscience) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Influenza virus strains including A/WSN/33 (H1N1), A/PR8/34 (H1N1) and A/HK/8/68 (H3N2), were propagated in 10-day-old embryonated chicken eggs. The allantoic fluids were harvested at 4 days after inoculation and stored in the freezer (-80 ^{o}C). For virus infections, confluent cells were incubated with diluted virus solutions in serum-free medium for 1 hour at 37 ^{o}C at the indicated multiplicities of infection (MOI). After the adsorption period, the viral inocula were removed and cells were washed twice with PBS (-), a Ca^{2+}/Mg^{2+} free-phosphate buffer solution. The cells were maintained in MEM or DMEM (supplemented with 0.5% FBS) containing 0.1% (v/v) DMSO or PGG at the indicated concentrations at 37 ^{o}C in a humidified 5% CO_2 incubator. 0.1% (v/v) DMSO was used as a vehicle control. For multiple replication of influenza A/PR8/34 and A/HK/8/68, the medium was additionally supplemented with 10μg/ml trypsin (Sigma-Aldrich).

2.3. WST-1 Assay

The cytotoxicity and cell-based antiviral activity of PGG were evaluated by Water Soluble Tertrazolium-1 (WST-1) assay [15]. For cytotoxicity analyses, confluent MDCK cells in 96-well plates (Greiner bio-one) were incubated with serial two-fold dilutions of PGG in MEM. Antiviral activity determinations were assessed in parallel. Serial two-fold dilutions of PGG were added to cells, followed by the addition of virus inocula of 100 $TCID_{50}$ (50% tissue culture infective dose) per well. After incubation for 72 hours, WST-1 (Dojindo Chemicals) solution (5 mM of WST-1 in 0.2 mM of 1-methoxy-5-methylphenaziniummethyl sulfate) was added to a final concentration of 0.25 mM. The optical density (OD) was measured 4 hours later by scanning at 450nm and 650nm reference wavelengths in the Emax precision microplate reader (Molecular Devices). Three independent experiments were carried out and each experiment was performed in triplicate. The percentage cell viability was compared with untreated controls and plotted against the compound concentration, and non-linear regression analysis was performed using Microsoft Excel software to calculate the 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}). The selectivity index (SI) for PGG was calculated by dividing the CC_{50} by EC_{50} (CC_{50}/EC_{50}).

2.4. Plaque forming unit assay (PFU assay)

The titers of infectious virus in culture supernatants harvested at the indicated time points were determined by PFU assay. Confluent MDCK cells in 6-well dish were infected with serial 10-fold

dilutions of the virus in a serum-free medium. After washing twice with PBS (-), cells were overlaid with MEM containing 0.8% (w/v) low melting agarose, 0.1% (w/v) BSA, 1% (v/v) vitamins, and 0.03% (w/v) glutamine. In the case of A/PR8/34 and A/HK/8/68, 10μg/ml trypsin was additionally supplemented. After 3 days of incubation, cells were fixed with ethanol: acetic acid (v/v=1:1) for 1 hour at room temperature and stained with 2.5% (w/v) Amino Black 10B after removal of the overlaying agarose gel. The plaques were counted by visual examination. Means and standard deviations were calculated from three independent experiments.

2.5. Time-of-addition assay

Time-of-addition experiments were performed in which PGG was added at different time intervals over a 24-hour incubation period. MDCK cells (6×10^5 cells/well) were seeded into 12-well plates and infected with virus at an MOI of 0.001. PGG (12.5 μg/ml) treatment or DMSO (0.1%, v/v) treatment was performed before, during, or after viral infection. At 24 hours post-infection (p.i.), culture supernatants of infected cells with different treatment were harvested and the virus titers were determined by PFU assay. The detailed procedures of each treatment were carried out as follows: (1) Pre-treatment of cells before virus infection: MDCK cells were pre-treated with PGG or DMSO and incubated at 37°C for 2 hours. After removal of the pre-treatment medium, the cells were washed twice with PBS (-), and infected with influenza virus. At 24 h p.i., cell supernatants were collected and virus yields were determined by the PFU assay. (2) Pre-treatment of virus before virus infection. The virus (3×10^3 PFU) was pre-incubated with PGG or DMSO on ice for 1 hour. The mixture of virus and PGG or DMSO was then added to MDCK cells, and incubated at 37°C for 1 hour. Cells were then washed twice and cultured in fresh medium for 24 hours. (3) Treatment of cells during virus infection. PGG or DMSO was administered together with the virus to the cells. After infection, cells were washed twice and cultured in fresh medium for 24 hours. (4) Treatment of cells after virus infection. After virus infection, cells were treated with PGG or DMSO at the indicated durations (as shown in Fig. 2a) and cultured in fresh medium for 24 hours.

2.6. Western Blotting

MDCK cells (2×10^5 cells/well) were seeded into 24-well plates, infected with virus (MOI=1), and followed by treatment with PGG (12.5 μg/ml) or DMSO (0.1%, v/v). At 0, 3, 6, 9 and 12 h p.i., cells were collected and lysed in sample buffer. An aliquot of 5 μl of each lysate was subjected to SDS-PAGE using a 10% separation gel. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) for Western blotting analysis, which was performed using mouse monoclonal antibodies against NP, HA and M1 proteins of influenza virus A (Santa Cruz Biotech) and α-Tubulin (Sigma-Aldrich). Bound antibodies were visualized with an enhanced chemiluminescence (ECL) Plus Kit (GE Healthcare Life Sciences).

2.7 Indirect immunofluorescence microscopy

MDCK cells were grown on glass coverslips and infected with virus (MOI = 3), and subsequently treated with PGG (12.5 μg/ml) or DMSO (0.1%, v/v) after infection. At the indicated time-points p.i., cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes and permeabilized with 0.02% Triton X-100 in PBS for 15 minutes, and then incubated with anti-NP monoclonal antibody

(Santa Cruz Biotech) for 1 hour at 37 °C. After washing with PBS, the cells were incubated with the goat anti-mouse IgG H & L chain specific biotin conjugate (Calbiochem) for 1 hour at 37 °C. Then streptavidin fluorochrome conjugates (Calbiochem) was added to the cells and incubated at 37 °C for 1 hour. Cell nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich) for 10 minutes at room temperature. Slides were examined under fluorescence microscope (Carl Zeiss) using 100× Plan Apo objective and the images captured with AxioVision software platform.

2.8. Transmission Electron Microscopy (TEM)

MDCK cells were infected with virus (MOI = 3) and treated with PGG (12.5 μg/ml) or DMSO (0.1%, v/v) for 12 hours, and then fixed in 3% glutaraldehyde (pH 7.2) for 1.5 hours, and post-fixed in 1% osmium tetroxide for 1 hour. After dehydration, cells were embedded in Spurr (Sigma-Aldrich). Several consecutive ultrathin sections were cut on an LKB Nova ultramicrotome (LKB) and then stained with saturated uranyl acetate and lead citrate. These sections were examined under a transmission electron microscope, JEM1400 (JEOL).

2.9. Quantitative real-time RT-PCR

MDCK cells (2×10^5 cells/well) were seeded into 24-well plates, and then infected with virus at different MOI (1 or 0.01), and followed by treatment with PGG (12.5 μg/ml) or DMSO (0.1%, v/v) for 12 hours. The culture supernatants were collected from infected cells, after removal of cellular debris by centrifugation, total RNA was isolated using PureLink™ Viral RNA/DNA Kits (Invitrogen) and reverse transcribed in the presence of random hexamers using a ReverTra Ace qPCR RT kit (Toyobo). The viral genomic segment 7 of influenza A/WSN/33 was specifically amplified by polymerase chain reaction (PCR) using the specific primers (sense: TCTGATCCTCTCGTCATTGCAGCAA; antisense: AATGACCATCGTCAACATCCACAGC). The cDNA was amplified by PCR using SYBR Green Real-time PCR Master Mix (Toyobo), as described by the manufacturer, using ABI PRISM 7000 Sequence Detection systems. The PCR conditions were as follows: denaturation at 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds/60°C for 1 minute. Melting curve analysis was performed to verify the specificity of the products. A standard curved (R^2 >0.99 within the range of 10^1-10^8 copies per reaction) was drawn to convert the respective cycle threshold (Ct) values into the number of viral genome copies. This standard consisted of a pCAGGS-WSN-M plasmid construct in which was cloned the full sequence of influenza virus A/WSN/33 segment 7. All samples were run in triplicate.

2.10 Hemagglutination inhibition (HI) assay

HI assay was carried out as described by Christina Ehrhardt et al.[10]. Briefly, serial two-fold dilutions of PGG (25μl) were prepared, mixed with equal volume of influenza virus solution (2^2 of HA units/25μl). After incubation of 1h at 4°C, 1% (v/v) of chicken erythrocytes (50μl) in PBS (-) were added and incubated for 30 min at room temperature.

2.11. Statistical analysis

The results were expressed as mean ± S.E.M. for the three independent experiments. Student's unpaired t-test was used to evaluate the difference between the test samples and untreated controls. A *P* value of <0.01 was considered statistically significant.

3. Results

3.1. Antiviral activity and cytotoxicity of PGG

For initial analysis of antiviral activity, PGG was tested on MDCK cells in the cell based screening assay. And the cytotoxicity of PGG was also evaluated. The EC_{50} value was 29.59 ± 4.32 μg/ml (31.48 ± 4.60 μM). No significant cytotoxicity was observed at concentrations of PGG up to 12.5μg/ml. PGG showed potent inhibitory activity against influenza A/WSN/33(H1N1), the EC_{50} value was 2.36 ± 0.29 μg/ml (2.51 ± 0.31 μM). The selectivity index (SI), which is expressed as the ratio of CC_{50}/EC_{50}, was 12.54.

3.2. Inhibitory effects of PGG on virus yield

To confirm the inhibitory effects of PGG on virus replication, virus yield was investigated in MDCK cells and A549 cells after infection with different strains of influenza A virus in the presence or absence of PGG. As shown in Fig. 1b, PGG significantly inhibited influenza A/WSN/33(H1N1) virus yields from MDCK cells at 24 and 48 h p.i. in a dose-dependent manner. Maximum reduction (over 4 $\log_{10}$ PFU/ml) was observed at concentration of 12.5 μg/ml. Comparison with the viral growth kinetics of PGG-treated cells and the DMSO-treated cells demonstrated that the inhibitory activity of PGG on virus yields remained stable during 48 h p.i. (Fig. 1c). In addition to influenza A/WSN/33(H1N1), PGG also inhibited the multiple replication of influenza A/PR8/34 (H1N1) and A/HK/8/68 (H3N2) in MDCK cells (Fig 1.d and e). The similar result was also observed in A549 cells instead of MDCK cells. Virus yield of A/WSN/33(H1N1) from A549 cells was inhibited under a multiple infection condition in the presence of 12.5μg/ml PGG (Fig.1 f). Over 80% of inhibition of virus yield was observed under a single infection of A/PR8/34 (H1N1) and A/HK/8/68 (H3N2) (Fig.1 g and h). These data indicated that PGG inhibits influenza A virus replication.

3.3. Mode of action of PGG against influenza A virus

To investigate the mode of action of PGG, time-of-addition experiments were performed (Fig. 2a). Pre-incubation of cells with PGG prior to infection showed no significant inhibitory effect on virus yield, however, a significant reductions (over 90%) in virus yield were observed when virus was pre-incubated with PGG prior to infection, or cells were treated with PGG during or after infection (Fig. 2b), suggesting that PGG may have virucidal activity. In order to determine whether PGG inhibited the virus yield during a specific period in virus replication cycle, the effect on compound addition at different time intervals using MDCK cells was studied. As shown in Fig. 2c, compared to DMSO treatment, even when PGG was added 12 hours later after infection, still a more than 95% reduction in virus yield was achieved during 24 hours of infection. Furthermore, to avoid an exposure of newly formed virions to PGG prior to titration, infected cells were exposed to PGG only within a single replication cycle (0-12 hours post-infection), and then the supernatants containing PGG or DMSO were replaced with fresh medium. As shown in Fig. 2d, in comparison to DMSO treatment, PGG treatment do not affect virus yield during the first 3 hours of treatment (0-3 hours), however, a significant reduction in virus yield was observed at 0-6 hours. Similarly, at 0-9 hours and 0-12 hours, the efficacy of inhibition reached 86% and 94%, respectively (Fig.2d). These results implicated that PGG may interfere predominantly with the late stage of the virus replication cycle independent of its viricidal

activity.

3.4. Inhibitory effects of PGG on hemagglutination

Since influenza A viruses are able to agglutinate chicken red blood cells (RBCs) by binding of their viral envelope spike protein hemagglutinin (HA) to the receptors on RBCs, to further confirm the effect of PGG on virus adsorption to cells, a hemagglutination inhibition assay was carried out. As shown in Table.1, PGG inhibited HA activity for all the three virus strains in a concentration dependent manner. These results suggested that PGG is capable of directly interacting with the viral glycoprotein HAs to block virus adsorption to cells.

3.5. Effects of PGG on viral protein synthesis

To confirm whether the inhibitory effects of PGG treatment on viral replication were related to the production of viral proteins, the expressions of viral proteins of infected cells, which were treated with PGG for 3, 6, 9 or 12 hours, were analyzed by Western blotting. As shown in Fig. 3, in which the viral protein levels were normalized by α-tubulin, PGG did not significantly affect the expressions of viral HA, NP and M1 proteins.

3.6. Effects of PGG on distribution of viral nucleoprotein (NP) in MDCK cells

To evaluate the effect of PGG on intracellular trafficking of virus ribonucleoprotein (NP), indirect immunofluorescence staining was performed using anti-NP antibody at 3, 6, 9, and 12 h p.i.. As shown in Fig. 4, viral NP accumulated in the nucleus of infected cells as early as 3 h p.i. (Fig. 4a and 4c), and translocation to the cytoplasm was completed within 9 h p.i. (Fig. 4i and 4k). No difference in the distribution pattern of NP was observed between PGG-treated and untreated cells until 6 h p.i.. The viral NP accumulated at the leading edge of cells without PGG treatment (Fig. 4i and 4m, arrow), but not in the PGG-treated cells at 9 and 12 h p.i. (Fig. 4k and 4o). These results indicated that PGG did not affect nuclear entry, or extranuclear translocation of NP at the early and middle stages of the replication cycle (0 to 6 hours), but interfered with the accumulation of NP on the surface of the cell membrane at the late stage of the replication cycle (9 to 12 hours).

3.7. Ultrastructural analysis of virus budding by TEM

Ultrastructural analysis of cells by TEM showed that most of the mock-infected cells were smooth-surfaced, or had sparsely scattered microvillar membrane protrusions (Fig. 5a and 5d). In contrast, many membrane protrusions were observed on the surface of infected cells that had not been treated with PGG (Fig. 5b and 5e), in which there were numerous budding viral particles (arrows) on the surface of the microvillar protrusions (Fig. 5g). PGG treatment reduced the appearance of microvillar protrusions on the surface of cells (Fig. 5c), and decreased budding virus particles (arrows), in which virus buds are seen lining the surface of the cell membrane (Fig. 5f). Virus buds exhibited a spherical shape rather than an elongated or filamentous form (Fig. 5h). These results revealed that PGG possibly affects the surface structure of the plasma membrane which may causes the reduction in virus assembly and budding on the surface of infected cells.

3.8. Effects of PGG on virus particle release

To confirm whether the release of total virus particles from infected cells was suppressed by PGG, we treated the infected cells with PGG within the first replication cycle (5h or 8h upon infection) and

determined the virus titers at 12h post-infection. As shown in Fig. 6 a and b, in comparison to DMSO treatment, treatment of PGG for 5h or 8h upon infection significantly inhibited the virus titer (over 90% reduction). We further analyzed the copy number of viral genomic RNA in the supernatants from infected cells at 12 h p.i. using quantitative real-time RT-PCR. A more than 70% of reduction in the amount of viral genomic RNA was observed in the culture supernatants in the presence of PGG (Fig. 6 c). These results indicated that PGG significantly suppressed total virus particle release in a single replication cycle.

4. Discussion

In the present study, our results indicate that PGG isolated from *Phyllanthus emblica Linn* effectively inhibits influenza A virus replication via two mechanisms: prevention of virus adsorption and suppression of virus release. Pre-treatment of virus before infection or treatment of cells during infection largely reduced virus yields during 24 hours of infection, however, pre-treatment of cells prior to infection did not significantly reduced virus yield, in addition, the early three hours of PGG treatment after infection did not affect on virus yield. Thereby, it is conceivable that the inhibitory effect of PGG was mainly caused by the direct interaction of PGG with virus. Numerous studies have demonstrated that plant polyphenols including the tea catechins (-) epigallocatechin gallate (EGCG) and theaflavin digallate [34], resveratrol (RV) [26], a polyphenol rich extract (CYSTUS052) [11], pomegranate polyphenol extract (PPE) [13] , oligonol[12], and Hydroxytyrosol (HT) [38], have potent antiviral activity against influenza virus, that are related to the nature of their interactions with viral particles. Additionally, indirect effects of some polyphenols, such as EGCG and strictinin, on host cells that might interfere with virus-cell membrane fusion have also been suggested [23, 32]. In this study, our results demonstrated that PGG inhibits virus-induced hemagglutination of chicken red blood cells, suggesting that PGG can interact with virus particles. Analysis of the detailed mechanisms of PGG acting on viral HA is currently underway.

Notably, our results also showed that PGG can reduce virus yields at the late stage of replication cycle independent of its virucidal activity. This effect has been suported by the results that virus release significantly reduced by PGG treatment in a single virus replication cycle. Reduced release of virus particles was evident in the results of four independent assays: (i) lower virus titers as determined by PFU assay, (ii) reduced membrane accumulation of NP protein as determined by immunofluorescence staining, (iii) decreased numbers of virus particles on the surface of plasma membrane as determined by TEM observation, and (iv) reduced viral genomic RNA in culture supernatants as determined by quantative realtime RT-PCR. Both treatment of PGG for 5h and 8h upon infection significantly inhibited virus release, suggesting that PGG may interfere with the steps before virus release in late stage of replication cycle. PGG treatment did not affect the expressions of viral proteins in infected cells and the nuclear transport of viral NP protein, but the accumulation of NP on plasma membrane was significantly suppressed in the presence of PGG, which was accordance with the reduction of virus buds on plasma membrane in the presence of PGG in TEM study. In addition, PGG treatment induced reduction of microvilli-like membrane protrusions, which is the site for virus assembly and budding. It is likely that PGG acts on the cellular membrane and therefore interferes with virus budding and release.

Influenza virus budding and release are essential for the transmission of the virus and in the pathogenesis of disease. A better understanding of these processes will help us in identifying new targets for prevention of influenza virus infection. Disruption of actin microfilaments by inhibitors alters the distribution of NP at the apical plasma membrane [33]. Influenza virus NP protein is known to associate with the actin cytoskeleton which may provide the pushing force for incorporating the vRNP complex into the bud [2], also vRNPs can be directed to the apical budding site via its

association with lipid rafts [4]. Although the molecular mechanism of transport of vRNPs to apical plasma membrane remains to be fully elucidated, recently, the Rab11-mediated membrane trafficking pathway has been reported to be required for IAV budding [3]. We have recently reported that PGG can down-regulate cofilin1, a key regulator of actin cytoskeleton dynamics, which might be associated with its anti-HSV-1 activity [28]. Whether these cellular factors as common targets were affected by PGG treatment needs be further examinated. Moreover, virus particles release from the surface of plasma membrane in the late stage of influenza virus replication requires the envelope spike glycoprotein neuraminidase (NA) that has sialidase activity [24]. The inhibition of virus release by PGG treatment whether associated with the effect of PGG on NA activity also seem to be an intriguing subject.

In conclusion, this study for the first time demonstrated that PGG possesses antiviral activity against influenza A virus *in vitro*. PGG inhibits productive replication of IAV not only by inhibiting virus infection but also by interfering with virus budding and release. The dual mode of action of PGG on virus observed in this study implies that PGG is a promising antiviral agent against influenza A virus.

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Figure legends

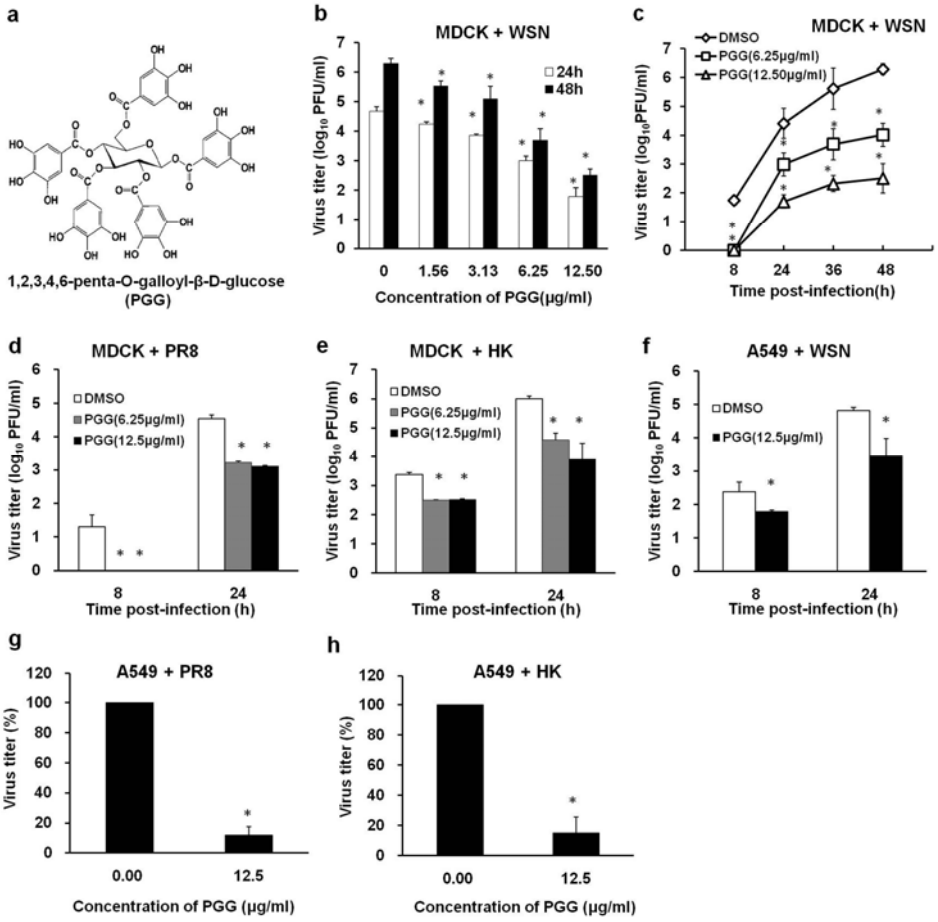

Fig.1 Inhibitory effects of PGG on viral yields. (a) Chemical structure of 1,2,3,4,6-penta-O-galloyl- β-D-glucose (PGG). (b) Dose-course of PGG treatment (1.56 to 12.5μg/ml) on virus titers. MDCK cells were infected with A/WSN/33 (MOI = 0.001), subsequently treated with serial 2-fold dilutions of PGG or 0.1% (v/v) of DMSO, culture supernatants were harvested at 24 and 48 hours post infection (p.i.), and virus titers were determined by plaque assay. (c) Growth kinetics of A/WSN/33 virus in MDCK cells. Cells were infected with A/WSN/33 virus (MOI = 0.001), and subsequently treated with PGG (6.25 and 12.5μg/ml) or DMSO (0.1%,v/v). Virus yields were determined at 8, 24, 36, 48h p.i.. (d) Effect of PGG on multiple replication of A/WSN/33 virus in A549 cells. Cells were infected with A/WSN/33 virus (MOI=0.01), and subsequently treated with PGG (12.5μg/ml) or 0.1% (v/v) of DMSO, virus yields was determined at 8 and 24h p.i.. (e) and (f) Effect of PGG on single step replication of A/PR8/34 and A/HK/8/68 virus in A549 cells. Cells were infected with A/PR8/34 virus and A/HK/8/68 virus (MOI=1), and subsequently treated with PGG (6.25 and 12.5μg/ml) or 0.1% (v/v) of DMSO, virus yields were determined at 8h p.i.. Values represent the mean of PFU/ml from three independent experiments and error bars show the standard deviation of the mean. The asterisks indicate significant difference between PGG and DMSO treatment, **P* < 0.01.

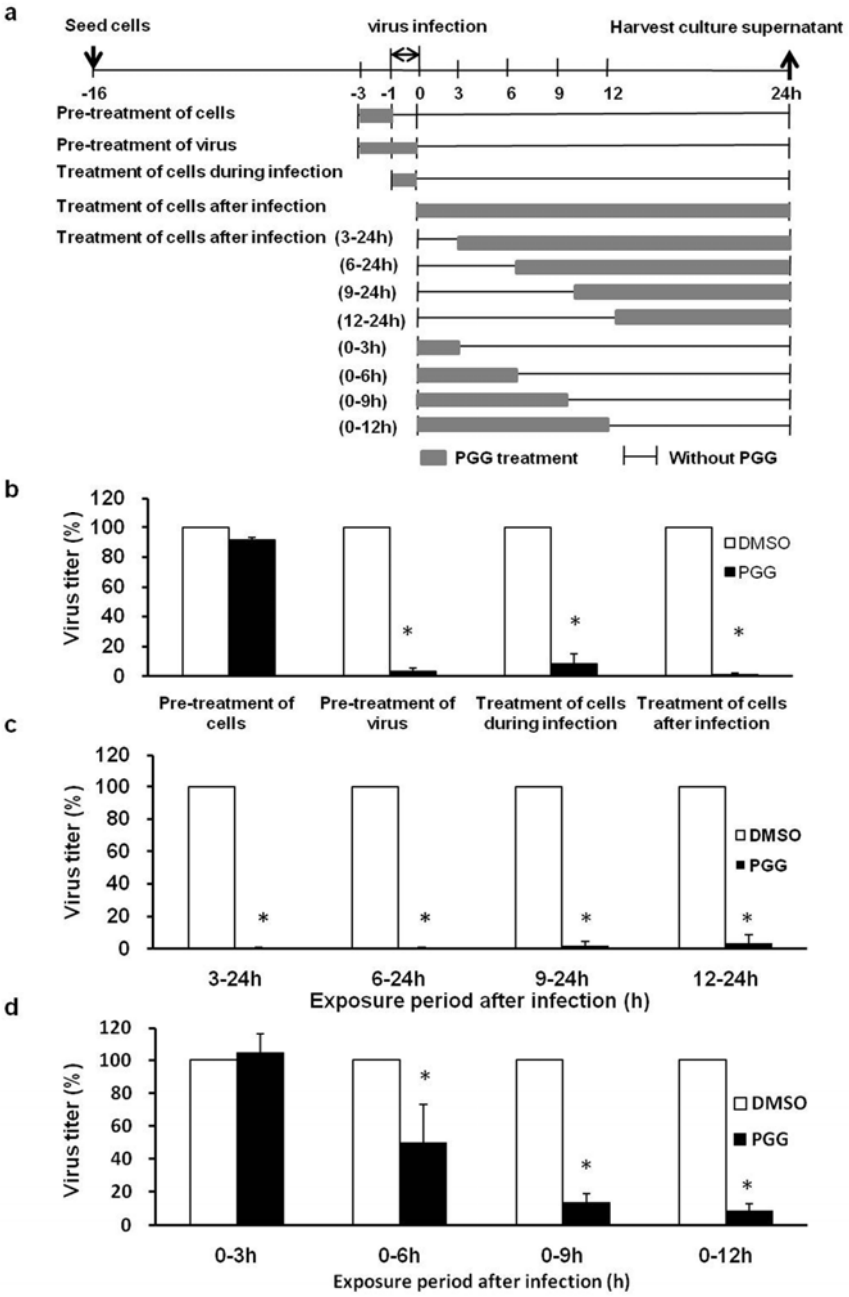

Fig. 2 Mode of action of PGG against influenza A virus. MDCK cells were infected with A/WSN/33 (MOI = 0.001) and treated with PGG (12.5 μg/ml) or 0.1% (v/v) DMSO (the control). The culture supernatant was collected at 24h post-infection, and virus yields were determined by plaque assay. (a) Different PGG treatment protocols. (b) PGG was added to cells before, during or after virus infection, or virus was pre-incubated with PGG prior to infection. (c) PGG was added to cells after virus infection at different time points (3h, 6h, 9h and 12h p.i). (d) Infected cells were exposure to PGG at different period time after infection (0-3h, 0-6h, 0-9h, 0-12h and 0-24h p.i). The virus titers from the PGG-treated cells were presented as a percentage of the control (treatment of DMSO (0.1%, v/v)). Values represent the mean of three independent experiments, and error bars show the standard deviation of the mean. The asterisks indicate significant differences between DMSO and PGG treatment, $*P < 0.01$.

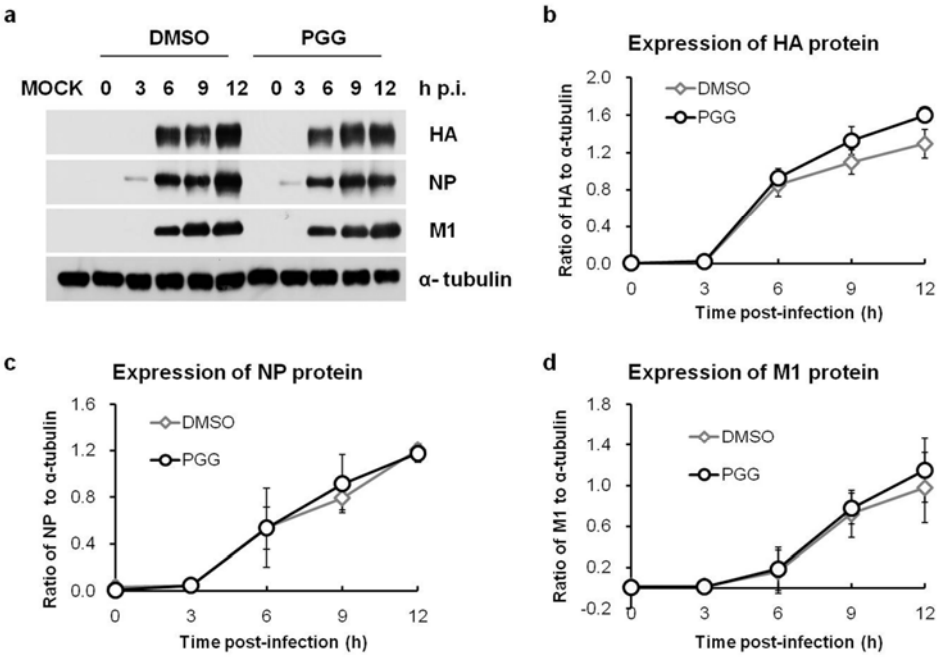

Fig. 3 Effects of PGG on viral protein synthesis. MDCK cells were infected with A/WSN/33 (MOI=1) and subsequently treated with 12.5 μg/ml PGG or 0.1% (v/v) DMSO for 12 hours after infection. At 0, 3, 6, 9 and 12 hours post-infection, cells were lyzed and subjected to Western blot analysis using monoclonal antibody against influenza A HA, NP and M1 proteins and anti-α-tubulin antibody(a). Protein band densities were quantified using Image J software. The relative levels of virus HA (b), NP (c) and M1 (d) protein expression were calculated by normalizing to that of α-tubulin, respectively.

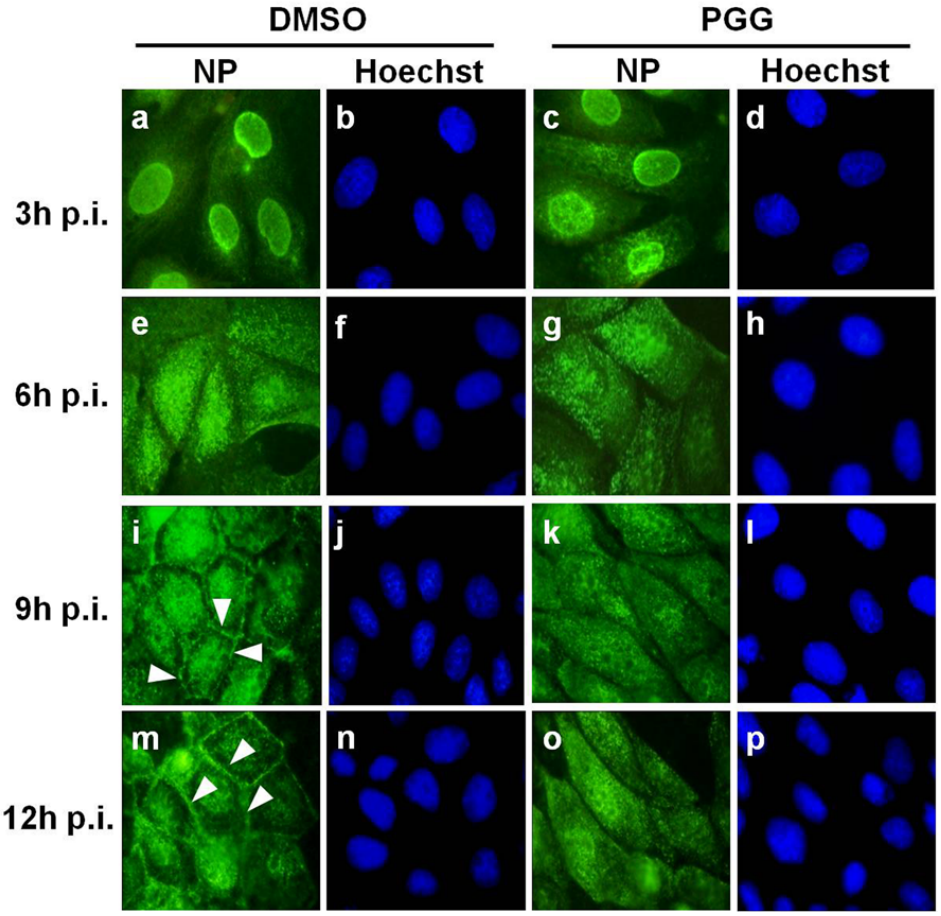

Fig. 4 Effects of PGG on subcellular distribution of viral nucleoprotein. MDCK cells were infected with A/WSN/33 (MOI = 3), and subsequently treated with 12.5 μg/ml PGG or 0.1% (v/v) DMSO after infection. Cells were fixed and immunofluorescence stained at 3 hours (a to d), 6 hours (e to h), 9 hours (i to l), and 12 hours (m to p) and analyzed by fluorescence microscopy. Cells were co-stained with anti-NP antibody (Alexa 546: Red) (a, c, e, g, i, k, m, o), and Hoechst 33342 (Blue) (b, d, f, h, j, l, n, p). The white arrows in i and m show the distribution of viral NP on the leading edge of cells.

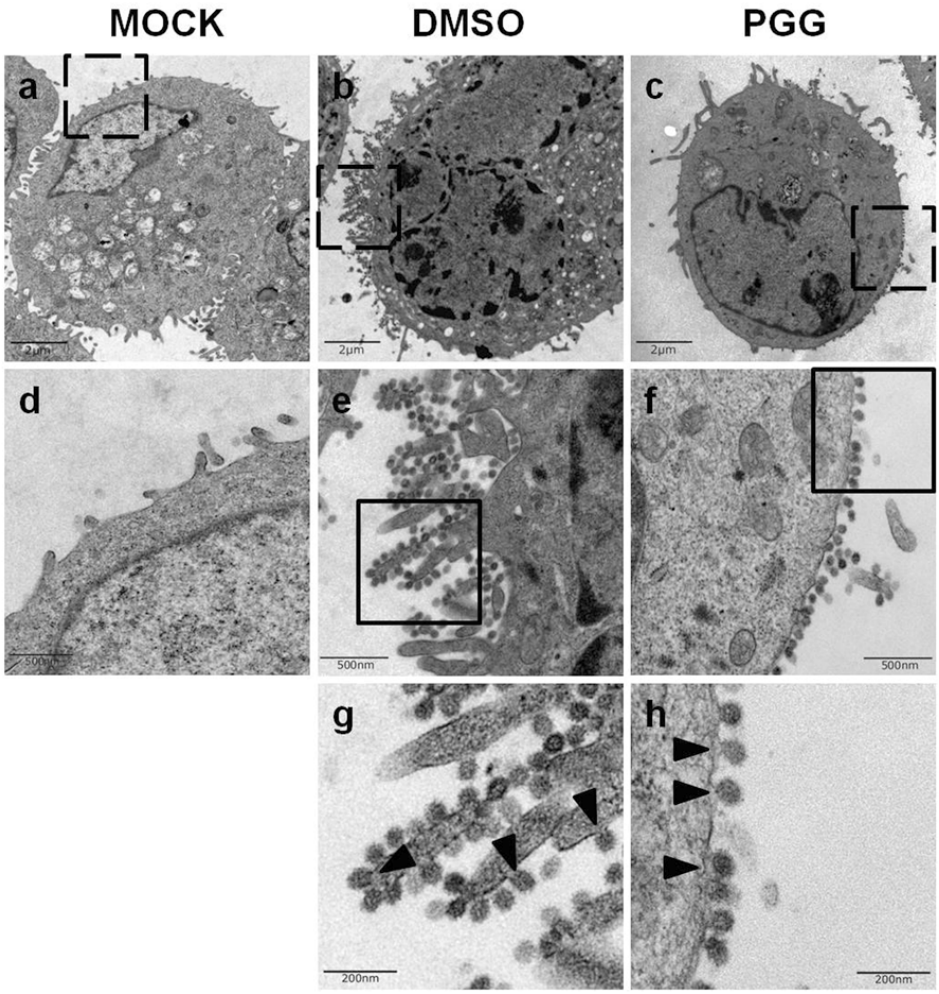

Fig. 5 Ultrastructure of virus budding on cell surface. MDCK cells were infected with influenza A/WSN/33 virus (MOI = 3), and subsequently treated with 12.5μg/ml PGG or 0.1% (v/v) of DMSO for 12 hours. Cells were examined with a TEM as described in Materials and Methods. The figure depicts mock-treated cells (a, d), infected cells treated with DMSO (b, e, g), and infected cells treated with PGG (c, f, h). Boxed areas with the dotted line are shown at a higher magnification in d, e and f. g and h represent the enlarged image of boxed areas with full lines in e and f, respectively. Arrows illustrate where virions are seen pinching off from the surface of the membrane.

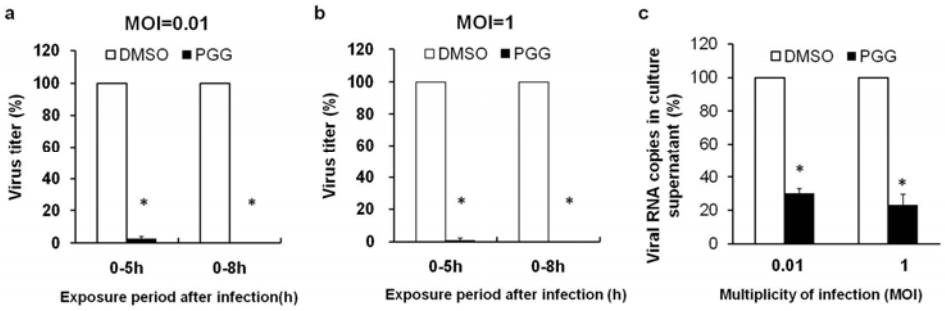

Fig. 6 Effects of PGG on viral particle release. (a) Inhibitory effects of PGG on virus titer. MDCK cells were infected with A/WSN/33 (MOI=1 and MOI=0.01), and treated with 12.5 μg/ml PGG or 0.1% (v/v) of DMSO for 5h or 8h upon infection. Virus titer of culture supernatant was determined at 12h post-infection. (b) Effects of PGG on the amount of viral RNAs in culture supernatant. MDCK cells were infected with A/WSN/33 (MOI=1, and MOI=0.01), and treated with 12.5 μg/ml PGG or 0.1% (v/v) of DMSO for 12 hours. Viral genomic RNA (segment 7) in culture supernatant was analyzed using quantitatively real-time RT-PCR. Viral RNAs in cell culture supernatants from PGG-treated cells were compared to the control (treatment with DMSO). Values represent the mean of three independent experiments, and error bars show the standard deviation of the mean. The asterisks indicate significant differences between DMSO and PGG treatment, $*P < 0.01$.

Table. 1 Inhibitory effects of PGG on hemagglutination

Strain	Negative Control	Positive Control	Virus + PGG (μg/ml)			
			1.56	3.13	6.25	12.5
A/WSN/33(H1N1)	–	+	+	–	–	–
A/PR/8/34(H1N1)	–	+	–	–	–	–
A/HK/8/68(H3N2)	–	+	+	+	–	–

+: indicates hemagglutination
– : indicates no hemagglutination
negative control : without virus
positive control : with virus