

The Anti-influenza Virus Effect of *Phellinus igniarius* Extract

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Herbal medicine has been used in the orient for thousands of years to treat large and small ailments, including microbial infections. Although there are treatments for influenza virus infection, there is no treatment for drug-resistant viruses. It is time that we explored and exploited the multi-component nature of herbal extracts as multi-drug combination therapies. Here, we present data on the anti-influenza virus effect of a medicinal mushroom, *Phellinus igniarius*. The *P. igniarius* water extract was effective against influenza A and B viruses, including 2009 pandemic H1N1, human H3N2, avian H9N2, and oseltamivir-resistant H1N1 viruses. Virological assays revealed that the extract may interfere with one or more early events in the influenza virus replication cycle, including viral attachment to the target cell. Therefore, our results provide new insights into the use of *P. igniarius* as an anti-influenza medicine.

Keywords: antiviral, herbal, influenza, multi-drug, *Phellinus igniarius*

Introduction

Influenza virus infection causes significant inconveniences in daily life, often hospitalization, and occasionally death (Neumann and Kawaoka, 2011). It can be prevented by seasonal vaccination, but circulating strains do not always match the vaccine strain (Tricco *et al.*, 2012; Pica and Palese, 2013). Once contracted, the influenza virus can be treated with neuraminidase inhibitors, such as oseltamivir, zanamivir, and peramivir, which are licensed globally or locally (Ison, 2011; Shetty and Peek, 2012). However, one problem arising from single-drug monotherapy is the generation of resistant strains; for example, treatment with the influenza virus M2 channel blocker, amantadine, and its derivatives has been terminated due to the development of widespread resistance (Pizzorno *et al.*, 2011). Additionally, unusual susceptibility against

antivirals is another problem associated with influenza treatment (Park *et al.*, 2012a).

The influenza virus genome consists of 8 segments of negative-sense, single-stranded RNA, which encodes at least 12 known proteins (Fields *et al.*, 2007; Muramoto *et al.*, 2013). The disturbance of any one of the proteins affects viral growth, host adaptation, and transmissibility. Theoretically, multiple drugs targeting multiple proteins are more effective and induce less resistance. Indeed, combinational therapy studies showed a synergistic effect (Ilyushina *et al.*, 2006; Govorkova and Webster, 2010; Nguyen *et al.*, 2010; Park *et al.*, 2012b).

A natural product extract can be considered a multi-drug combination therapy in the sense that multiple components in an extract may potentially target multiple viral proteins. Natural herbal extracts are important therapeutic methods in oriental medicine (Zhu *et al.*, 2008). Although undefined, natural, nontoxic combinations of multiple anti-influenza components could be the most effective measure against influenza. Therefore, we aimed to screen natural extracts for anti-influenza activity, which led us to *Phellinus igniarius*.

Mushrooms of the *Phellinus* genus are well-established ingredients in oriental medicine (Sliva, 2010). The most studied medicinal fungus is *P. linteus*, which is reported to inhibit tumor growth (Song *et al.*, 2008, 2011) and immune modulation (Song *et al.*, 1995; Hwang *et al.*, 2012). *Phellinus igniarius* is another representative medicinal fungus, the extracts of which have anti-proliferative (Yang *et al.*, 2006) and anti-metastasis (Song *et al.*, 2008) effects. Glycans are the major components of mushroom extracts. They vary according to the extraction method used (Yang *et al.*, 2009) and are the reported agents of therapeutic effects (Meng *et al.*, 2012). We postulated that the mushroom extracts interfere with influenza virus binding to the target cell surface, which involves glycan receptor recognition (Fields *et al.*, 2007). The *P. igniarius* extract reportedly inhibits cell proliferation, and it may also inhibit viral polymerases. Here, we report the antiviral effect of a *P. igniarius* water extract (PIW) against influenza viruses and discuss the potential mechanism(s) of the antiviral effect.

Materials and Methods

Preparation of PIW and oseltamivir carboxylate

The crude powder of dried fruit bodies of *P. igniarius* (Linnaeus: Fries) Quélet 1886 (Amazing Grace Health Product, Bangkok, Thailand) was boiled at 103°C in distilled water for 3 h. The aqueous extract was mixed with two volumes of cold (-20°C) 95% ethanol and then stored in a refrigerator overnight. Dark brown precipitate was collected after centrifugation and freeze-dried. The extract was loaded on an open column (8 cm × 45 cm) of HP-20 (Supelco, USA) and

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sequentially fractionated with water, 25% ethanol, and 50% ethanol. Each fraction was freeze-dried. Oseltamivir carboxylate was purchased from Toronto Research Chemicals Inc. (Canada) for use in cytotoxicity and replication inhibition assays.

Cells and viruses

Madin-Darby canine kidney (MDCK) and human lung epithelial (A549) cells were grown in Eagle's minimum essential medium (Lonza, Switzerland) and Dulbecco's modified Eagle medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37°C in 5% CO₂. The recombinant PR8 virus expressing green fluorescent protein (GFP) (rPR8 NS1-GFP; provided by Dr. Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai, USA) (Manicassamy *et al.*, 2010), A/Korea/01/2009 (2009 pandemic H1N1 virus, K09), X-31 (H3N2 vaccine virus), A/Chicken/Korea/01310/2001 (H9N2 virus, Ck01; provided by Dr. Young Ki Choi, Chungbuk National University in Cheongju, Korea), human B (clinical isolate in Korea in 2011, B11), and seasonal H1N1 (clinical isolate in Korea in 2008 harboring an NA H275Y mutation, S08) viruses were purified by a standard plaque assay in MDCK cells and propagated in 10-day-old embryonic chicken eggs.

Virucidal assay of PIW against rPR8 NS1-GFP and K09 viruses

The rPR8 NS1-GFP virus (MOI=0.5) was pretreated with different PIW concentrations (0.125–2 mg/ml) for 1 h at room temperature using a routine infection procedure. Briefly, confluent monolayers of MDCK cells in 96-well plates were inoculated with the mixture. After a 1-h incubation period, the mixture was removed. Cells were washed with phosphate buffered saline (PBS) three times and supplied with culture medium. After 24 h at 37°C in 5% CO₂, GFP signals were

observed under a fluorescence microscope. For the K09 virus, approximately 200 plaque-forming units (PFU) were pretreated with the same PIW concentrations prior to the plaque assays.

Replication inhibition assay of PIW against the rPR8 NS1-GFP virus

Confluent monolayers of MDCK cells in 96-well plates were infected with the rPR8 NS1-GFP virus (MOI=0.5) and supplemented with 2-fold serially diluted PIW (final concentration of 0.125–2 mg/ml). After 24 h, GFP signals were observed under a fluorescence microscope.

Determination of the 50% inhibition concentration (IC₅₀) of PIW against the K09 virus

The K09 virus (approximately 200 PFU) was incubated with different concentrations of PIW (0.125–2 mg/ml) for 1 h at 37°C in 5% CO₂ prior to performing a routine plaque assay. The IC₅₀ value was calculated from triplicate results using Prism 5.0d (Graphpad software, USA) and was determined as the concentration that reduced the number of plaques to 50% of the non-treated control value.

Hemagglutination inhibition (HAI) assay

Chicken red blood cells (cRBCs) were washed three times with PBS. PIW (20 mg/ml) in serial 2-fold dilutions in 25 µl of PBS was mixed with an equal volume (25 µl) of the K09 virus (4 HAU) and incubated for 1 h at 37°C. The 50-µl solution was mixed again with an equal volume of 0.5% (v/v) cRBC suspension. After 30–45 min, HAI activity was determined by the amount of hemagglutination present.

Cell cytotoxicity assay

PIW cytotoxicity was measured by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-

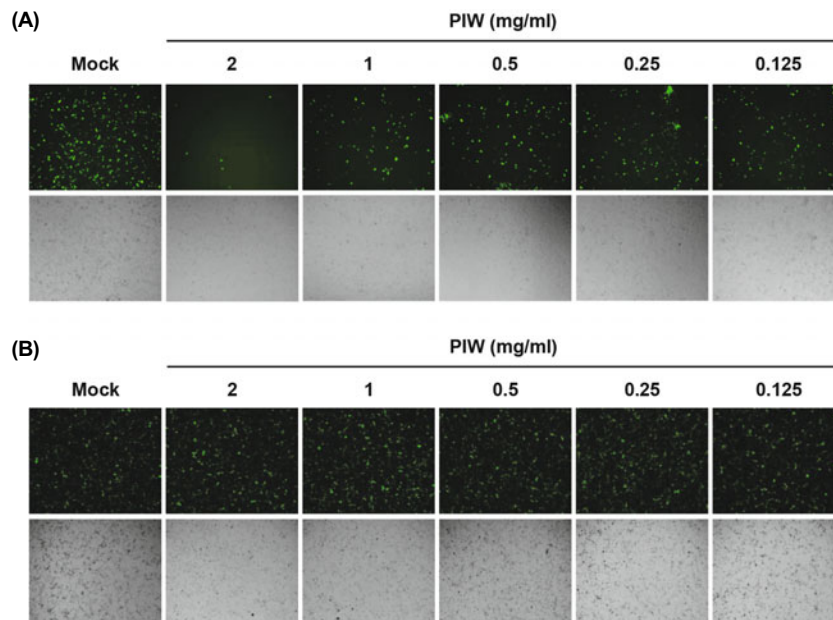


Fig. 1. Inhibitory efficacy of PIW against the rPR8 NS1-GFP virus. (A) The rPR8 NS1-GFP virus (MOI=0.5) was pretreated for 1 h with different concentrations (0.125–2 mg/ml) of PIW. The PIW-treated virus samples were then used to infect MDCK cells. (B) Alternatively, the same concentrations of PIW were added to the cell culture media instead of directly applying the PIW to the rPR8 NS1-GFP virus (MOI=0.5). After 24 h, images were taken using a fluorescence microscope. GFP images are shown in the upper panels, and phase images of the cells are shown in black and white in the lower panels. PBS, instead of PIW, was used for the mock control.

Aldrich, USA) assay. Briefly, MDCK or A549 cells were grown in 96-well plates for 24 h. The medium was replaced with medium containing 2-fold serially diluted PIW (starting from 50 mg/ml), and the cells were incubated for another 24 h. The medium was removed, and 50 μ l of MTT solution (2 mg/ml in PBS) was added to each well. The plates were incubated at 37°C for 1 h. After removal of the MTT solution, 50 μ l of DMSO was added, and the culture was incubated for 10 min. The absorbance was measured at 540 nm in an ELX800-UV ELISA reader. The 50% cytotoxicity concentration (CC_{50}) value was calculated as the concentration that reduced the number of viable cells to 50% of the non-treated control value.

Replication inhibition assay in cells

A549 cells were treated with PIW at different concentrations (0.125–2 mg/ml) for 18 h, followed by cell washing. The rPR8 NS1-GFP infection was performed by a routine procedure. After 24 h at 37°C in 5% CO_2 , GFP signals were observed under a fluorescence microscope. To evaluate the inhibition of K09 virus replication, single- (MOI=2) or multi-replication (MOI=0.01) virus samples were pretreated with PIW (500 μ g/ml). MDCK cells were infected with each sample and maintained in media supplemented with PIW (500 μ g/ml). At the indicated time points, cell supernatants were collected for virus titration via plaque assay. Oseltamivir carboxylate (50 μ g/ml) was used as a control.

Results

PIW anti-viral effects on PR8 NS1-GFP influenza virus replication

The anti-influenza virus effect of PIW was brought to our attention by a screening of natural product extracts against an influenza virus expressing GFP (Kim *et al.*, 2012). Pre-treatment of the rPR8 NS1-GFP virus with PIW for 1 h at room temperature before infection resulted in a PIW concentration-dependent reduction of the GFP signal in MDCK cells (Fig. 1A). However, when PIW was added to the infection culture medium after virus adsorption to the target cell, a reduction in GFP-expressing cells was not observed (Fig. 1B). Therefore, PIW was likely an antagonist of influenza virus adsorption during the first hour of infection. The data also suggested that PIW did not inhibit influenza infection after virus entry into the cell and that PIW did not block viral polymerase function.

The PIW anti-influenza mechanism against the 2009 pandemic influenza H1N1 virus

Because the rPR8 NS1-GFP virus used in the screening was a recombinant virus based on an H1N1 subtype, laboratory strain PR8 virus, we next determined whether PIW could inhibit a 2009 pandemic influenza H1N1 virus (pH1N1), namely A/Korea/01/2009 (K09). Similar to the rPR8 NS1-GFP virus, pre-treatment of the K09 virus with PIW resulted

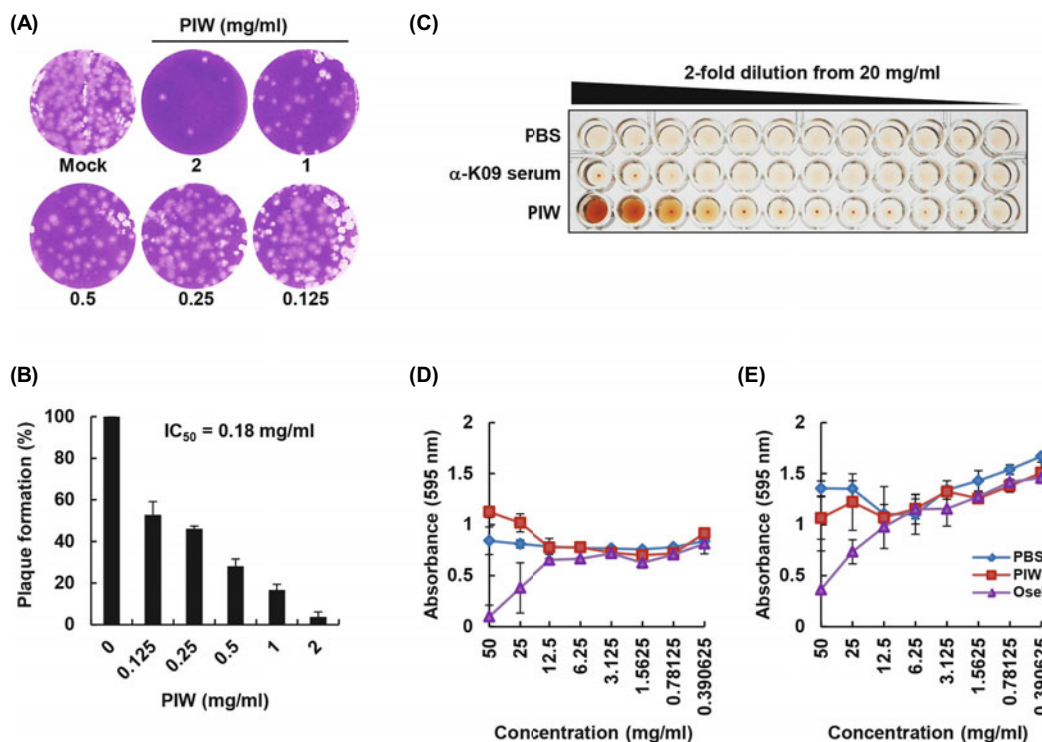


Fig. 2. PIW cytotoxicity and inhibition of the pH1N1 K09 virus. (A) Approximately 200 PFU of the K09 virus were pretreated with different concentrations of PIW (0.125–2 mg/ml) prior to the plaque assay. (B) Triplicate results were plotted for IC_{50} calculations. (C) In the HAI assay, the PIW-mediated inhibition of K09 virus adsorption to chicken red blood cells was assessed. PBS was used as a negative control, and anti-K09 guinea pig serum (α -K09) was the positive control. (D, E) The cytotoxicity of PIW was examined in MDCK (D) and A549 (E) cells using an MTT assay. The results were determined by three independent experiments. Error bars denote standard deviations (SDs). PBS and oseltamivir carboxylate were used as controls.

Table 1. PIW IC₅₀ values for various influenza viruses

Virus	Strain	IC ₅₀ (mg/ml) ^a
K09	2009 pandemic H1N1	0.18
S08	H1N1 harboring NA H275Y	0.36
X-31	H3N2 vaccine virus	1.14
B11	Human B	0.99
Ck01	Avian H9N2	0.56

^a PIW IC₅₀ values were determined using 200 PFU of each virus in triplicate virucidal assays. For detailed information, see the 'Materials and Methods' section 'Determination of the 50% inhibition concentration (IC₅₀) of PIW against the K09 virus'.

in a concentration-dependent reduction (Fig. 2A). The IC₅₀ of PIW against the K09 virus was 0.18 mg/ml, as calculated from triplicate plaque assay results (Fig. 2B). In the rPR8 NS1-GFP virus replication inhibition assay, we observed that PIW was ineffective when it was added after virus adsorption (Fig. 1B). A plaque reduction assay revealed the conditions under which plaque number and size were reduced during multi-cycle replication; this standard assay determines the inhibitory effect of a chemical against a virus. As expected from the inhibitory effect of PIW on the infection of the pretreated virus, we observed a PIW concentration-dependent reduction in plaque number and size (data not shown). In terms of the mechanism(s) of inhibition by PIW, the inhibitory effect observed in a single replication cycle of pretreated virus strongly suggested that PIW affected the viral life cycle within 1 h of virus attachment to the cell. We used HAI assays to determine whether PIW affected virus attachment to the target cell receptor. Influenza viruses are characterized by their ability to agglutinate erythrocytes (Fields *et al.*, 2007). This hemagglutination activity can be visualized upon mixing virus dilutions with cRBCs in 96-well plates. As shown in Fig. 2C, PIW exhibited HAI activity against the K09 virus at a concentration as low as 20 mg/ml. This result suggested that inhibiting influenza virus attach-

ment to the cell surface receptor is one mechanism of PIW action.

Broad-spectrum anti-influenza efficacy and cytotoxicity of PIW

The previous results suggested that PIW blocked virus entry. Although all influenza viruses bind to the terminal sialic acid of glycans, human influenza viruses bind primarily to sialic acid linked to galactose by α -2,6, and avian influenza viruses bind to α -2,3-linked sialic acid (Connor *et al.*, 1994; Stevens *et al.*, 2006). To determine whether PIW activity was limited to human H1N1 subtypes, we measured the IC₅₀ values of PIW against the human H3N2-subtype vaccine strain X-31 (Kilbourne, 1969; Shil *et al.*, 2011), avian influenza subtype H9N2, field-isolated influenza B, and oseltamivir-resistant H1N1 strains (Table 1). PIW was most effective against the pH1N1 K09 virus. Interestingly, although both the X-31 and K09 viruses have human receptor binding specificity, the PIW IC₅₀ against the X-31 virus (1.14 mg/ml) was approximately 6 times that against the K09 virus (0.18 mg/ml). The pronounced HAI activity of PIW against K09 (Fig. 2C) was not observed with the X-31 virus and other viruses (data not shown), which partly explains the higher efficacy of PIW against the K09 virus and suggests that other mechanism(s) are involved in PIW-mediated inhibition. Cytotoxicity assays revealed that the PIW CC₅₀ was higher than 50 mg/ml on MDCK cells (Fig. 2D) and human A549 cells (Fig. 2E). In summary, PIW was effective against the K09 virus and against all other viruses tested, including S08, X-31, B11, and Ck01.

Replication kinetics of viruses in PIW-treated cells

Because PIW was present during adsorption of the pretreated virus to the target cell or during the HAI assay, PIW could have altered target cell susceptibility to virus entry. We next

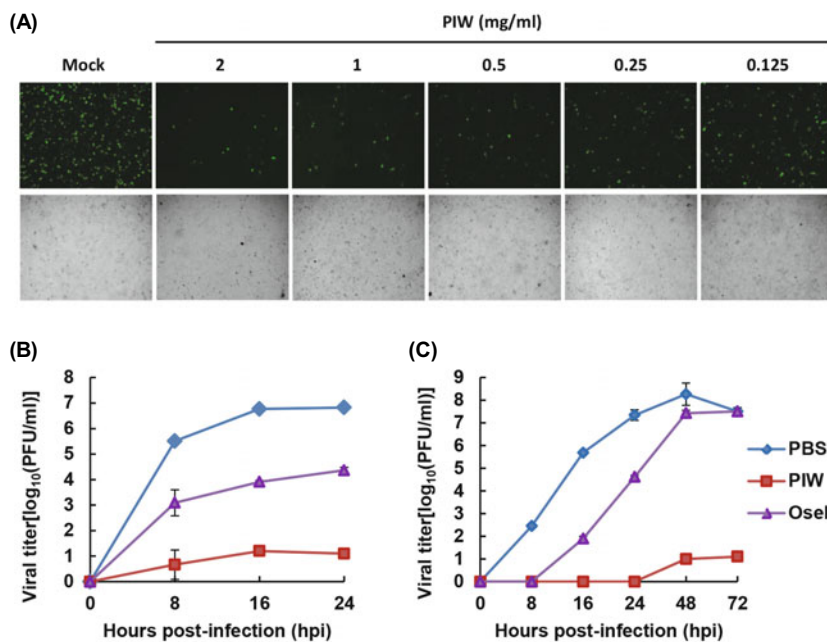


Fig. 3. Replication of rPR8 NS1-GFP and K09 viruses in PIW-treated cells. (A) A549 cells were treated with different concentrations of PIW (0.125–2 mg/ml) for 18 h, and the cells were infected with the rPR8 NS1-GFP virus (MOI=0.5). After 24 h, the cells were observed under a fluorescence microscope. GFP images are shown in the upper panels, and phase images of the cells are shown in black and white in the lower panels. PBS, instead of PIW, was used for the mock controls. (B, C) After the PIW pretreatment (500 µg/ml) with the K09 virus (B, MOI=2; C, MOI=0.01), MDCK cells were infected with the virus samples and maintained in media supplemented with PIW (500 µg/ml). At indicated time points, cell supernatants were collected for the virus titration via plaque assays. The results were determined by three independent experiments. Error bars denote SDs. PBS and 50 µg/ml oseltamivir carboxylate were used as controls.

asked whether pretreating the target cells with PIW blocked influenza virus infection. To visualize the effect of pretreatment of A549 cells with various concentrations of PIW on influenza virus infection, we again used the rPR8 NS1-GFP virus. The GFP signals in Fig. 3A showed that PIW pretreatment inhibited virus infection in a PIW concentration-dependent manner. The phase images of A549 cells show that the 18-h pretreatment did not adversely affect the cells (Fig. 3A).

The anti-influenza effect observed in cells was assessed against the single- or multi-replication kinetics of the K09 virus. In MDCK cells, the K09 virus exhibited extremely reduced growth rates in both single- (Fig. 3B) and multi-replication (Fig. 3C) inhibition assays compared with oseltamivir carboxylate-treated controls. Because adding PIW to the infection culture medium did not have any inhibitory effect, PIW pretreatment may have affected the cell membrane surface and/or the cell function(s) associated with virus entry. The pretreated cells may not have been able to support virus attachment during receptor binding or the subsequent step of endocytosis (Luo, 2012).

Discussion

Water extracts from natural products, prepared according to traditional medicinal practices, have proven to be safe and balanced compositions of multiple active, therapeutic components. Thus, natural product extracts should be explored for their potential as resistance-proof treatments for human viruses and especially RNA viruses, which are notorious for developing resistance to antiviral drugs (Chen *et al.*, 2005; Wargo and Kurath, 2012). We have shown that PIW has potential as an anti-influenza medicine. Our data suggest that PIW is effective against diverse influenza virus subtypes within the human and avian influenza A and B viruses. Mechanistically, PIW may affect influenza virus infection by interacting with both the virus and the target cell.

Virus attachment to the cell surface, endocytosis of the virus, and fusion of viral and endosomal membranes resulting in the release of viral genomes into the cytoplasm are very early steps in influenza virus replication (Martin and Helenius, 1991). These processes may have been completed within the 1-h virus adsorption period when virus plus PIW were incubated with the target cells (Fig. 1A). The PIW component(s) could have bound to the virus and affected any of these early steps in viral replication. As shown in Fig. 1B, PIW did not interfere with viral polymerase activity. However, the HAI assay clearly showed that PIW interfered with influenza virus attachment to the cell surface (Fig. 2C). As indirectly confirmed by HAI activity, the virus is a potential target of PIW action in the case of the K09 virus (Fig. 2C) but not X-31 and other viruses (data not shown). Because hemagglutinin (HA) from different viruses has varying affinity for the receptor (Kim *et al.*, 2013), PIW may not have interfered with the interaction between the receptor and X-31 HA, which has the same receptor specificity as the K09 virus. Against X-31 and other viruses (Table 1), PIW may exert a different effect that does not involve the inhibition of receptor binding.

Because cells pretreated with PIW were protected from influenza virus infection (Figs. 3A, 3B, and 3C), we conclude that PIW has component(s) that bind to the target cell and interfere with the early replication step(s) of the virus. The effect of PIW against the K09 virus may have been at least partly due to preoccupation of the HA receptor binding site by one or more components of PIW. For other viruses unaffected by PIW, glycans in PIW (Yang *et al.*, 2009) may have interfered with the lectin-mediated binding of the virus to the cell (Yang *et al.*, 2011; Hillaire *et al.*, 2013). Reciprocally, lectins in PIW could have bound to the cell surface glycans and blocked virus binding to the receptor glycans, but the presence of lectins in *Phellinus* mushroom extracts has not been reported. Other component(s) of PIW may have bound to the cell surface or intracellular factor(s) involved in endocytosis, blocking virus endocytosis (Mooren *et al.*, 2012), or to other viral or cell membrane component(s) that are critical for membrane fusion (Derby and Gleeson, 2007; Schroeder, 2010), thus preventing virus fusion to the endosome. All of these possibilities need further investigation, although they are beyond the scope of the current work.

The current study presents a framework for exploring natural product extracts as natural, multi-drug, combination antiviral therapies, specifically against the influenza virus. We used GFP-mediated screening to evaluate PIW as a potential anti-influenza medicine, and we reported the nature and potential mechanism of PIW activity against influenza infection through stepwise virological characterizations. Our data provide the basis for further mechanistic study. Furthermore, we found that prolonged pretreatment of the target cell with PIW did not adversely affect the cells and that pretreated cells were protected against influenza virus infection. These results prompt the practical exploration of using PIW as a preventive treatment during the influenza season.

Acknowledgements

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