Influenza Virus Entry and Replication Inhibited by 8-Prenylnaringenin from *Citrullus Lanatus Var. Citroides* (Wild Watermelon)

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Abstract

We previously demonstrated the anti-influenza activity of *Citrullus lanatus var. citroides* (wild watermelon, WWM); however, the active ingredient was unknown. Here, we performed metabolomic analysis to evaluate the ingredients of WWM associated with antiviral activity. Many low-molecular-weight compounds were identified, with flavonoids accounting for 3% of all the compounds in WWM juice. Prenylated flavonoids accounted for 13% of the flavonoids. Among the ingredients, 8-prenylnaringenin exhibited the highest antiviral activity. We synthesized 8-prenylnaringenin and used liquid chromatography-mass spectrometry to quantitate the active ingredient in WWM. The antiviral activities of 8-prenylnaringenin were observed against H1N1 influenza subtypes, including oseltamivir-resistant H1N1 viruses, but not against an influenza B virus. Moreover, 8-prenylnaringenin was found to inhibit virus adsorption and late-stage virus replication, suggesting that the mechanisms of action of 8-prenylnaringenin may differ from those of amantadine and oseltamivir. This is the first report on the anti-influenza virus activity of 8-prenylnaringenin. Our results highlight the potential of WWM in developing effective prophylactic and therapeutic approaches against influenza viruses.

Introduction

Influenza is an acute respiratory infection caused by the influenza virus (IFV), which belongs to the family Orthomyxoviridae and is prevalent worldwide. Types A, B, and C IFV can infect humans with types A and B influenza, causing seasonal epidemics every year and sometimes causing severe complications, such as pneumonia and encephalitis [1]. Vaccines and antiviral drugs are used to prevent and treat IFV infection, respectively. However, these vaccines fail to induce a stable preventive effect [2]. In addition, the emergence of IFV strains resistant to amantadine and oseltamivir has become a serious problem in recent years [3, 4]. Thus, a novel approach to protect against IFV infection is needed.

Recently, functional foods showing antiviral activity have been reported [5, 6, 7], and ingredients of functional foods have received increased attention. Some foods have been reported to have various ingredients with anti-INF activity, including tea polyphenols such as catechins, theaflavins, and procyanidins [8]. Catechins in green tea [9, 10] showed neuraminidase inhibitory activities and IFV growth inhibitory effect through acidification of the intercellular compartment [11]. In addition, green tea suppressed inflammation, cell proliferation, and apoptosis through the regulation of the nuclear factor kappa B (NF-κB), an important transcriptional regulator [12]. It has also been suggested that cocoa polyphenols and anthocyanin pigments in hibiscus tea exhibit anti-IFV activity [13, 14]. All the components of adlay tea, adlay seeds, naked barley seeds, soybean, and cassia seeds inhibited both IFV adsorption and virus replication, resulting in strong antiviral activity against influenza A H1N1 and H3N2 subtypes and influenza B viruses [7, 15]. The anti-IFV activity of soybean daidzein differs from that of oseltamivir and functions via signal transduction through 5-lipoxygenase products [16].

Citrullus lanatus var. citroides, commonly known as wild watermelon (WWM), can adapt and grow under severely dry and high-ultraviolet-light conditions and is native to the Kalahari desert in southern Africa. In

its native region, WWM is used as a dietary source of hydrogen and a water source to wash the body. WWM has a high citrulline content, which protects the plant from the stresses of its native environment [17, 18], and the seeds contain many essential amino acids [19]. Although there have been several reports on the usefulness of WWM, its food functionality remains a relatively new area of research. In a previous study, we reported an anti-influenza activity of WWM juice, but the effective components remained unknown [6]. In the current study, we aimed to investigate the flavonoid-based components present in WWM juice and evaluate the activity of prenylated flavonoids against IFV replication. Specifically, we focused on prenylated naringenins because naringenin from *Citrus junos* has been previously shown to inhibit influenza A virus [20], and prenylated polyphenols have been shown to accumulate in Caco-2 intestinal epithelial cells and hepatocytes, with their intracellular concentration being 60 times higher than the extracellular concentration [21].

Materials And Methods

Compounds

All reagents used for chemical synthesis not explicitly mentioned were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industry Co. (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), and Sigma-Aldrich Co. (St. Louis, MO, USA). (±)-Naringenin was purchased from Cayman Chemical Ltd and dissolved in dimethyl sulfoxide (DMSO) as a stock solution (50 mg/mL). Meanwhile, (±)-8-prenylnaringenin (8-PN) was synthesized from (±)-naringenin in a four-step process with a 24% overall yield according to a previously reported procedure [22] and as detailed in the supplementary methods. In the current study, (±)-naringenin was used instead of (*S*)-naringenin, considering the cost.

Cells and viruses

MDCK cells were grown in EMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 7% FBS. In the current study, we used influenza A H1N1 strains A/PR/8/34, A/Suita/114/2011, A/Osaka/2024/2009, and A/Osaka/71/2011; H3N2 strains A/Sydney/5/97, and A/Aich/2/68; and B strains B/Shanghai/261/2002 and B/Nagasaki/1/87. Treatment of the cells against viral infections was according to the method by Morimoto et al. [6]

Metabolomic data analysis

The metabolomic data was obtained via LTQ ORBITRAP XL analysis (Thermo fisher scientific) using the Power Get software (http://www.kazusa.or.jp/komics/ja/tool-ja/48-powerget.html) originally developed by the Kazusa DNA Research Institute [23]. Chromatographic separation was performed at 40 °C using a TSK gel ODS-100V column (3mm×50mm, 5μm: TOSOH) on an Agilent 1200 series system. For separation, the mobile phases were optima grade water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A 25-min gradient at a flow rate of 0.4 ml/min with the following conditions was used: 0–5 min, held at 1% B; 5–10 min, linear gradient from 1–3% B; 10–18 min, linear gradient from 3–40% B; 18–22 min, linear gradient from 40–80% B; 22–27 min, column cleaning at 95% B; and 27–35

min, re-equilibration with solution A. The injection volume was 5 µl, and the MS was operated in the positive ion mode (ESI) with a scan range of m/z 100–1500 using one of the top five MS/MS methods. The average accurate mass of the compound peak was collated with a public database (Flavonoid Viewer) using Kazusa DNA Research Institute development Software (MF Searcher)

LC/MS measurement (triple-quadrupole, QQQ)

LC/MS measurement was performed according to a previously described method [24]. Analysis was carried out according to a previously described method [25].

Cell viability determination

Cell viability was performed according to the method by Morimoto et al. [6] using a Cell Proliferation Kit I (MTT) (F. Hoffmann–La Roche Ltd, Basel, Switzerland).

Antiviral assay of 8-PN

The effects of the addition of the compounds on viral yield were determined as previously described [7], with slight modifications. MDCK cells were cultured in 24-well plates (Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 1×10^5 cells/well in 500 ml/well of EMEM containing FBS and incubated for 24 h at 37 °C. In case of adsorption inhibition, diluted viruses were allowed to infect confluent cells at an MOI of 0.01 for 1 h at 37 °C with or without 11.4 µg/ml 8-PN. After 1 h of adsorption, infected cells were rinsed once with serum-free EMEM and then cultured in DMEM containing 0.4% BSA (500 µl/well) without 8-PN. After 8 h, the infected cells as IFV samples were frozen at -80 °C and subjected to two freeze-thaw cycles prior to determining the viral yield by focus-forming assays. In the case of replication inhibition, diluted viruses were allowed to infect the cells at an MOI of 0.001 for 1 h at 37 °C. After 1 h of adsorption, the infected cells were rinsed once with serum-free EMEM and then cultured in DMEM containing 0.4% BSA (500 µl/well) with or without 11.4 µg/ml 8-PN. After 24 h, the supernatants were collected as IFV samples and subjected to focus-forming assays.

Focus-forming reduction assay (FFRA) for measuring virus titer

FFRA was performed as previously described [6], which is a modification of another previously described method [7].

Time-of-addition assay

We conducted a time-of-addition experiment using a previously described procedure [6] with slight modifications. The difference was the concentration of the inhibitor, 8-PN. DMEM containing 0.02 mg/ml of the compounds, which was approximately 80% the maximum inhibitory concentration (Fig. 1), was added at different periods of infection: during adsorption, for 1 h incubation with viruses; during replication for up to 8 h, measured every two- and four-hour intervals (Fig. 2a). The infected cells were

then frozen at -80 °C 8 h after infection and subjected to two freeze-thaw cycles before determining the viral yield using the focus-forming assay.

Viral binding inhibition assay The viral amount attached to the cells was determined by measuring the viral RNA encoding the HA protein (HA) using SYBR green and a pair of primers, HA-F: 5'-TTGCTAAAACCCGGAGACAC and HA-R: 5'-CCTGACGTATTTGGGCACT. Viral RNA bound to cells was extracted, and cDNA was synthesized; viral RNA was quantified as described previously [7]. As a normalization gene for real-time PCR based on influenza virus-infected cells, 18S rRNA was quantified as described previously [26].

Results

Metabolomic data analysis of WWM juice

We conducted a metabolomic analysis to identify the active components in WWM juice, focusing on flavonoids that have been reported. Many low molecular weight compounds were identified (1,646), including 578 different flavonoids that comprised 35% of the total compounds present in the WWM juice (Table 1). There were 228 glycosylated flavonoids and 350 aglycons. Thus, the proportion of aglycons compared to all flavonoids detected was 61%. The WWM juice contained 173 prenylated flavonoids, which accounted for 30% of the detected flavonoids, and 172 of the prenylated flavonoids were aglycons. Some of the prenylated flavonoids detected are shown in Table 2.

Table 1
Classifying polyphenols based on backbone structure

Backbone structure	Numbers
Aurone and Chalcone	83
Flavanone	99
Flavone	182
Dihydeoflavonol	23
Flavonol	90
Flavan	6
Anthocyanin	0
Isoflavonoid	91
Neoflavonoid	4

Table 2
Classifying polyphenols based on ornamentation groups

Glycosylation	Modification	Numbers
No	no	91
No	alkylated	12
No	prenylated	172
No	furano flavonoid	22
No	pyrano flavonoid	53
O-glycoside	no	120
O-glycoside	alkylated	1
O-glycoside	prenylated	1
O-glycoside	furano flavonoid	1
O-glycoside	prenylpropanoid	8
C-glycoside	no	62
O-&C-glycoside	no	18
Others		17

Quantification of 8-PN in WWM juice

The antiviral activity of one of the prenylated flavonoids, 8-PN, was measured, and the results are summarized in Table 1. We focused on prenylated naringenins, such as 8-PN (Fig. 1a), which was detected by liquid chromatography-mass spectrometry measurement (QQQ) at approximately 1.5 ng/g in the WWM juice. The chemically synthesized 8-PN strongly inhibited viral growth in MDCK cells (Fig. 1b). Moreover, it showed no evidence of cytotoxicity at concentrations lower than 25 μg/ml (Fig. 1c). We then evaluated viral replication in MDCK cells treated with naringenin and 8-PN. Both naringenin and 8-PN inhibited IFV growth in a concentration-dependent manner, but the virus growth inhibition activity of 8-PN was approximately 10 times higher than that of naringenin (data not shown). The IC_{50} values of naringenin and 8-PN were 70 and 5.5 μg/ml, respectively.

The critical steps targeted by 8-PN

The stage of viral replication inhibited by 8-PN was identified using time-of-addition assays. Fig. 2a shows the periods at which 8-PN was included in the incubation mixture. As reported previously [7], one replication cycle of A/PR/8/34 within a cell takes approximately 8 h. Based on this information, the

stages of viral multiplication blocked by 8-PN were elucidated, as demonstrated in Fig. 2b. We then segmented the exposure period to 8-PN during viral replication into 2-h intervals. The results revealed that two different steps of the virus infection process were inhibited by 8-PN. The first step was viral adsorption to the cells (−1 to 0 h of viral infection). The second step was during actual viral replication, specifically the late stage of replication (4–8 h post-infection), and especially, the period associated with viral assembly (6–8 h post-infection). Thus, the time-of-addition assay showed that 8-PN blocks at least two stages of viral growth, adsorption and late replication (Fig. 2b).

Viral adsorption inhibition by 8-PN

8-PN restricted viral entry in all type A and type B IFVs evaluated, including oseltamivir-resistant viruses such as A/Osaka/2024/2009 and A/Osaka/71/2011 (Table 3). This suggested that the mechanism of action of the ingredients in WWM may differ from that of amantadine [4]. A/Osaka/2024/2009 and A/Osaka/71/2011, which are H1N1 2009 pandemic (pdm09) viruses, and type B viruses are amantadine resistant [3, 4].

The addition of 8-PN inhibited viral adsorption (Table 3) in a temperature- and concentration-dependent manner (Fig. 2c), indicating that 8-PN affects viral endocytosis. The mechanism of adsorption inhibition was not due to the interaction between 8-PN and viral components but may have resulted from the interaction between 8-PN and cell components via signal transduction, similar to that of daidzein [16].

Table 3. Effect of 8-prenylnaringenin on the multriplication of various influenza virus strains

	Adsorption		Replication	
Virus type and strain	IC_{50} (μg/mL)	SI[a]	IC_{50} (μg/mL)	SI[a]
A (H1N1)				
PR/8/34	18.4 ± 3.1	3.6	5.5 ± 0.4	12.2
Suita/114/2011	12.3 ± 2.6	5.4	24.3 ± 1.1	2.8
Osaka/2024/2009[b]	6.4 ± 2.1	10.5	ND[c]	ND[c]
Osaka/71/2011[b]	14.3 ± 2.1	4.7	ND[c]	ND[c]
A (H3N2)				
Sydney/5/97	7.1 ± 0.7	9.4	2.9 ± 1.6	23.1
Aich/2/68	6.7 ± 0.6	10.0	2.5 ± 0.2	26.8
B				
Shanghai/261/2002	11.7 ± 0.3	5.7	2.2 ± 0.4	30.4
Nagasaki/1/87	12.3 ± 0.2	5.4	6.6 ± 0.2	10.1

Viral replication inhibition by 8-PN

Regarding the inhibition of replication, 8-PN inhibited all type A and type B IFV, except for oseltamivir-resistant viruses, such as A/Osaka/2024/2009 and A/Osaka/71/2011 (Table 3). This implies that the mechanism of action of 8-PN may be the same as that of oseltamivir.

Meanwhile, the inhibition of late replication may have been associated with viral neuraminidase as 8-PN did not inhibit the viral replication of oseltamivir-resistant viruses (Table 3). Therefore, the 8-PN viral replication inhibition mechanism may be the interaction between viral neuraminidase and 8-PN, similar to that of oseltamivir.

Discussion

Like various polyphenols, naringenin also shows anti-influenza activity [15, 16, 20, 27]. It was hypothesized that the anti-influenza effect of flavonoids might stem from their ability to coordinate metal ions, as documented by various quercetin-metal ion complexes reported in the literature [28, 29].

Prenylation of polyphenols not only creates a new affinity for membranes [30] but may also affect permeability. In cell experiments, there is also a report that a prenylated polyphenol, xanthohumol, is concentrated 60-fold in cells [21]. In addition, the prenylated polyphenol is bound to cellular proteins, which may alter the properties of cellular factors [21]. Thus, it has been suggested that prenylated polyphenols may be involved in intracellular signal transduction and enzymatic and physiological activities. Furthermore, a wide range of bioactivities, such as the prevention of osteoporosis and anticancer activities, are known for prenylated polyphenols, such as 8-PN [31]. Daidzein, known as phytoestrogen, exhibited anti-influenza activity by activating cells at the late replication stage [16], but this is the first report on anti-influenza activity of 8-PN on the two stages. Notably, the mechanisms of action of daidzein and 8-PN were found to be different.

The time-of-addition assay (Fig. 2b) showed that 8-PN blocks at least two stages of viral growth, adsorption and late replication. Regarding adsorption inhibition, 8-PN restricted viral entry of all type A and type B IFVs evaluated, including amantadine- and oseltamivir-resistant viruses (Table 3). H1N1 2009 pandemic (pdm09) viruses and type B viruses are amantadine-resistant [3, 4]. This suggested that the mechanism of action of the WWM ingredients may differ from that of amantadine [4]. IFVs are internalized via receptor-mediated endocytosis [32], and inhibition of endocytosis may effectively prevent infection. For instance, resveratrol, a natural polyphenol, reduces the internalization of cholera toxin by inhibiting its endocytosis into the cells [33]. As adsorption inhibition by 8-PN was shown to be temperature-dependent, 8-PN may also exert its activity by inhibiting endocytosis of IFV.

Regarding replication inhibition, 8-PN inhibited all type A and type B IFV, except for oseltamivir-resistant viruses such as A/Osaka/2024/2009 and A/Osaka/71/2011 (Table 3). This implies that the mechanism of action of 8-PN may be the same as that of oseltamivir. Although 8-PN failed to show antiviral activity against oseltamivir-resistant viruses, WWM juice exhibits antiviral activity against these viruses [6]. This suggests that WWM contains additional ingredient(s) with antiviral activities that affect the replication of oseltamivir-resistant viruses, similar to the activity of daidzein [16].

The current study has shown a hitherto unknown anti-IFV activity of 8-PN. However, because the levels of 8-PN in WWM are inadequate to exert the observed antiviral activity, other antiviral ingredients were likely involved. As the antiviral effect of WWM is probably a combined effect of several ingredients, further studies are needed to identify the other active ingredients and establish the precise mechanisms of action.

This study evaluated the anti-IFV activity of the ingredients of WWM, which were detected by metabolome analysis, and demonstrated antiviral activity by 8-PN. The ingredient(s) inhibited the viral adsorption and late replication stages in the growth process of IFVs. Our results also indicate that the antiviral mechanism of 8-PN against IFV growth during virus adsorption may differ from that of amantadine, while the mechanism of endocytosis and late replication inhibition may be similar to that of oseltamivir. This is the first report of the anti-IFV action of 8-PN. Furthermore, the study findings highlight the potential role of WWM in the development of novel prophylactic and therapeutic approaches against influenza.

Declarations

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Competing interests

The authors declare no competing financial interests.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Consent for publication: Not applicable

Authors' contributions

AH and RM performed the experiments, collected the test data, and interpreted the results. YH and MS performed the experiments and designed and supervised the PCR assay. AN, TO, and KS provided the materials and performed the data collection for the metabolomic analysis of WWMJ. HS analyzed the components of WWMJ and wrote the MS-chromatography part of the Methods section. TO synthesized 8-PN and wrote the chemical synthesis part of the manuscript. YI conceptualized the study design, supervised the whole study, and wrote the main manuscript. All authors reviewed the manuscript.

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Figures

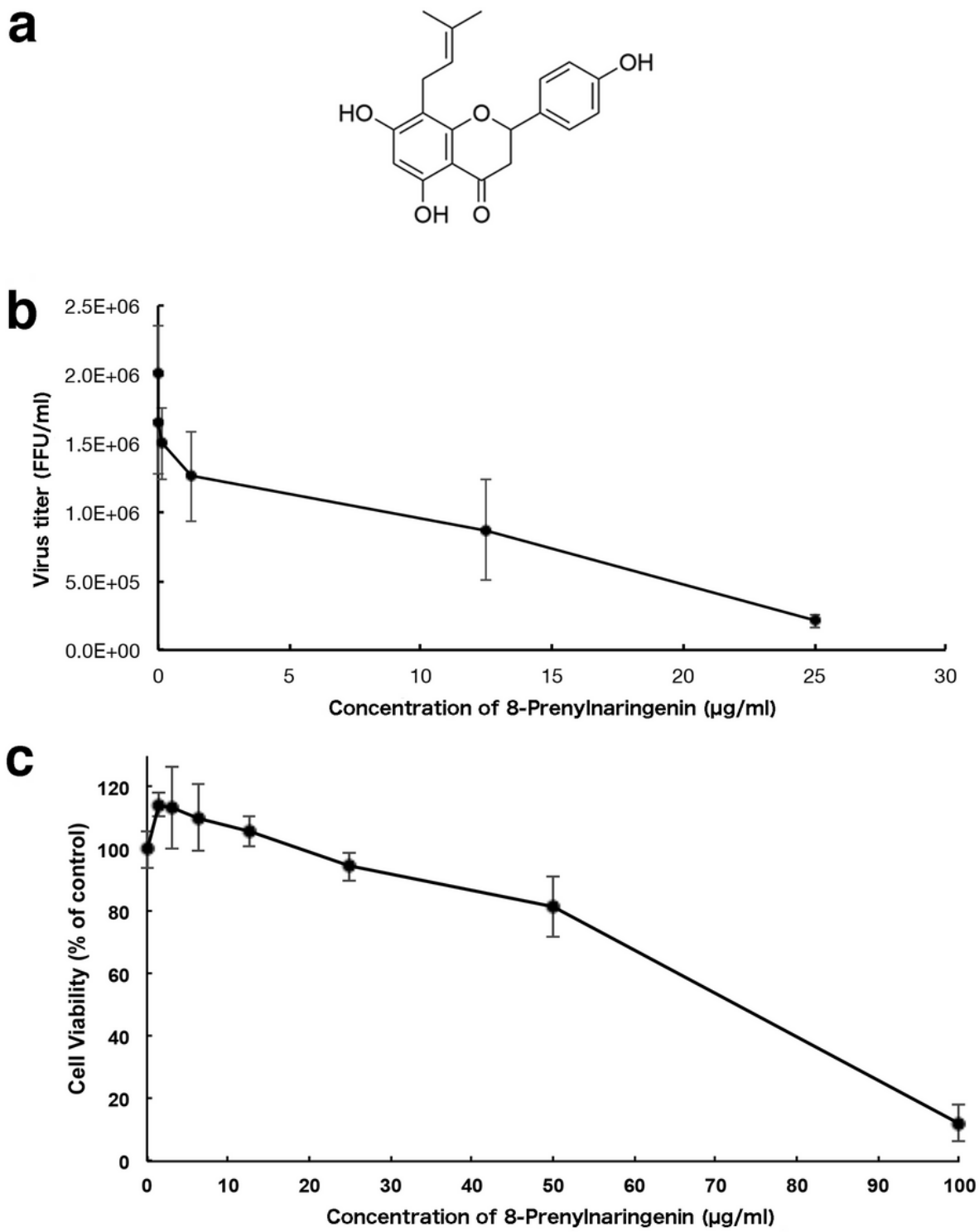

Figure 1

8-PN inhibited H1N1 influenza virus replication in MDCK cells. MDCK cells were incubated with influenza A/PR/8/34 virus at a multiplicity of infection of 0.001. Viral yields were determined 24 h post-infection using focus-forming assays. Vertical lines indicate standard deviation (n = 3). (a) Structure of 8-PN. (b) Concentration-dependent inhibitory effect of 8-PN compounds on virus multiplication. The virus titer was

determined 24 h post-infection using focus-forming assays. (c) Cytotoxicity of 8-PN compounds. Vertical lines indicate standard deviations (n = 3). Data are representative of three independent experiments

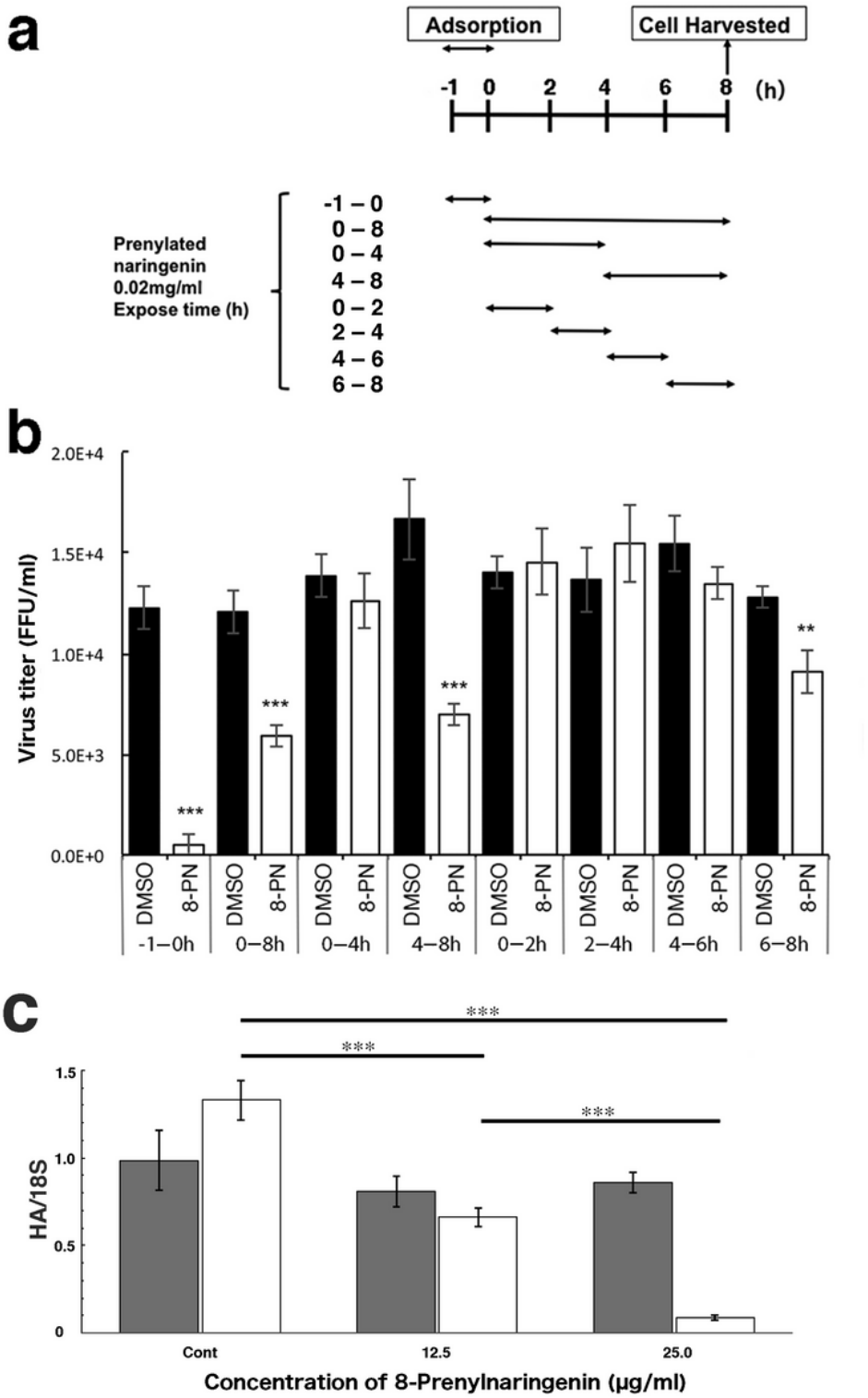

Figure 2

Effect of 8-PN on viral replication. MDCK cells in 24-well plates were infected with influenza virus A/PR/8/34 at a multiplicity of infection of 0.01. DMEM containing 8-PN (0.02 mg/mL: more than twice the IC50 value) was then added at the different times shown. (a) Time-of-addition assay schedule. (b)

After infection according to the indicated schedule, the cells were harvested, and viruses were assayed using focus-forming reduction assays (FFRAs). Filled columns, mean viral yields of control cells; open columns, mean viral yields of cells treated with 8-PN. Data are representative of three independent experiments. (c) Effect of 8-PN on viral binding to cells. Closed columns indicate viral adsorption onto cells at 4 °C at three different concentrations of 8-PN; open columns, 37 °C. Error bars indicate SD (n=3). Data are representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001

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