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Research Article

The Anti-Influenza Virus Effect of Acacetin by Inhibiting Clathrin-Dependent Endocytosis and RNA Synthesis

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Abstract

Influenza viruses are a public health concern as they cause seasonal and pandemic infections. Although vaccines and antiviral drugs are available, limited vaccine efficacy and the emergence of drug-resistant strains highlight the need for new treatment strategies. Acacetin, a common bioactive component in *Mosla scabra* and *Isatis tinctoria* L., inhibits viral entry. However, its mechanisms of action during the entry, early culture, and late culture phases of virus propagation remain unclear. Herein, we investigated the mechanism by which acacetin inhibits viral proliferation. Fluorescence microscopy of viral nucleoproteins showed that acacetin inhibited viral uptake into cells, with viruses remaining localized on acacetin-treated cell membranes compared with that in control cells. Transferrin and cholera toxin assays examining the effects of acacetin on the clathrin- and caveolae-dependent pathways showed that acacetin inhibited viral uptake into cells via clathrin-dependent endocytosis. Analysis of viral RNA expression levels at each viral growth stage in acacetin-treated cells showed that acacetin inhibited viral RNA replication and transcription. Therefore, acacetin is a potential antiviral drug candidate with mechanisms of action distinct from those of existing drugs. Future studies should identify additional antiviral compounds in *M. scabra* and *I. tinctoria* and elucidate the mechanisms by which acacetin inhibits clathrin-dependent endocytosis.

Keywords: Influenza Viral Infection Inhibition; Clathrin-Dependent Endocytosis; RNA Synthesis

Abbreviations

BSA	:	Bovine Serum Albumin
CTxB	:	Cholera Toxin B
DAPI	:	4′,6-diamidino-2-phenylindole
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide
FBS	:	Fetal Bovine Serum
HA	:	Hemagglutinin
HRP	:	Horseradish Peroxidase
IC_{50}	:	Half Maximal Inhibitory Concentration
MDC	:	Monodansylcadaverine
MDCK	:	Madin–Darby Canine Kidney
MEM	:	Minimum Essential Medium
NA	:	Neuraminidase
NP	:	Nucleoprotein
rRNA	:	*ribosomal RNA*
TBS-T	:	Tris buffered saline with Tween 20
RIPA	:	Radio-Immunoprecipitation Assay
MOI	:	Multiplicity of Infection

Introduction

Influenza virus infection is a respiratory infection that causes seasonal epidemics yearly and has a serious impact on human health [1]. Infection and mortality rates are particularly high in the elderly, young children, pregnant women, and those with certain chronic diseases. These groups are also more likely to develop complications, such as pneumonia, secondary bacterial infections, and multiple organ failure [2,3]. Influenza viruses are RNA viruses belonging to the Orthomyxoviridae family and are classified into types A, B, C, and D. Subtypes are further classified based on the antigenic characteristics of Hemagglutinin (HA) and Neuraminidase (NA) present in the virus envelope [1].

Currently, as a countermeasure against infectious diseases caused by the influenza virus, vaccination is used for prevention and antiviral drugs are used for treatment [1,2]. However, these measures have some limitations. Prevention through vaccination is problematic owing to its low efficacy. HA and NA of the influenza virus spike proteins easily undergo mutations, resulting in new antigenic variants [4]. Furthermore, new strains can be generated by the crossover of gene segments between different influenza viruses [4]. Therefore, to develop vaccines, it is necessary to predict the antigenicity of viruses that are prevalent annually. Furthermore, developing vaccines against new strains is time-consuming. Therefore, it is difficult to completely prevent all viral infections using vaccines.

The emergence of drug-resistant strains has made antiviral drug treatment challenging. Amantadine, introduced into clinical use in 1966, is an antiviral drug that blocks the M2 ion channel of influenza A viruses, thereby inhibiting viral shedding and the nuclear entry of viral RNA (vRNA) [4]. However, it is ineffective against influenza B viruses, which lack the M2 protein, and by 2013, many influenza A viruses circulating worldwide (69% of H1N1 and 43% of H3N2) were resistant to amantadine [5]. Therefore, amantadine is no longer recommended for influenza treatment. In contrast, oseltamivir and zanamivir, currently widely used as therapeutic drugs for influenza viruses, competitively inhibit NA, which acts on replicated viruses inside cells. However, among the influenza A viruses (H1N1) tested in the United States during 2008–2009, resistance to oseltamivir was confirmed in 98.5% of cases [6]. In India in 2015, the oseltamivir-resistant influenza A(H1N1)pdm09 virus was associated with high case fatality and was not observed among non-fatal cases [7].

With the emergence of resistant strains, several vRNA polymerase inhibitors have been recently developed. Baloxavir marboxil (trade name: Xofluza), a cap-dependent endonuclease inhibitor, was approved in 2018; however, the emergence of resistant strains has already been reported [8]. Additionally, T705 (product name: Avigan), which was approved in 2014, inhibits the RNA replication of a wide range of RNA viruses; however, it has been conditionally approved in Japan owing to its teratogenicity and embryotoxicity, and is not recommended for pregnant women and children [9]. Therefore, preventing and treating infections caused by influenza viruses is challenging. To overcome these challenges, new vaccines, prevention methods, and antiviral drugs that have different effects from those of existing drugs are required.

Anti-influenza virus activity of food components, such as epigallocatechin gallate [10], fucoidan [11], sulforaphane [12], quercetin [13], daidzein [14], and acacetin [15-17], has been reported. Our laboratory has demonstrated anti-influenza virus activity from soybeans [18], Kalahari watermelon [19], pearl barley, naked barley, and quince [18]. Furthermore, we have elucidated the relationship between the structure and antiviral activity of various flavonoids [20].

Mosla scabra (Thunb.), a plant used as an antipyretic and antiviral remedy for lung diseases in southeastern China, is effective against flu, fever, inflammation, and chronic bronchitis. Four compounds, 5-hydroxy-6,7-dimethoxyflavone, 5-hydroxy-7,8-dimethoxyflavone, apigenin, and acacetin, showed significant anti-influenza virus activities [16]. *Isatis tinctoria* L. (Banlangen), a traditional herbal medicine widely used in Asian countries, is substantially effective against respiratory viruses. Six compounds, eupatorin, dinatin, linarin, tryptanthrin, indirubin, and acacetin,

showed inhibitory activities against influenza viruses [15]. According to Morimoto et al. [20], acacetin, an active flavonoid, is the common bioactive substance in both plants. Acacetin suppresses viral invasion and replication by inhibiting endocytosis [17]. However, the host factors through which acacetin exerts its anti-influenza virus effect during the entry, early culture, and late culture phases of virus propagation are still unclear. Therefore, this study aimed to clarify the detailed mechanism underlying the anti-influenza virus activity of acacetin and its interactions with host factors.

Materials and Methods

Cells and viruses

Madin–Darby canine kidney (MDCK) cells (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) were cultured in minimum essential medium (MEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 7% fetal bovine serum (FBS; Biowest Biotechnology Company, Nuaillé, France). HeLa cells were maintained in Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS at 37 ˚C in a 5% CO_2 atmosphere. The influenza A virus H1N1 (PR/8/34; Research Institute for Microbial Diseases, Osaka University; Osaka/2024/2009 and Osaka/71/2011, Osaka Institute of Public Health, Osaka, Japan) was used. To investigate the inhibitory mechanism during the late stage of viral multiplication, we tested viral activity using oseltamivir-resistant strains. These strains are resistant to NA inhibitors, such as oseltamivir and zanamivir, which suppress NA activity during viral budding [21]. Viruses were added to MDCK cells at a multiplicity of infection (MOI) of 0.001, adsorbed in a CO_2 incubator (Panasonic Healthcare, Osaka, Japan) at 37 ˚C for 1 h under 5% CO_2 conditions, incubated with 2 μg/mL acetyltrypsin (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and DMEM (Sigma-Aldrich) containing 0.4% bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Corporation) in the 5% CO_2 incubator, and allowed to grow in culture [A(H1N1): 24 h]. After incubation, the supernatant was collected and stored as virus solution frozen at -80 ˚C until use. The viral stock was used within three freeze-thaw cycles.

Sample preparation

Acacetin was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc., Kyoto, Japan) at a concentration of 14.2 mg/mL (50 mM) and stored at –30 ˚C. Bafilomycin A1 (AdipoGen Life Sciences, Inc., San Diego, CA, USA), a V-ATPase inhibitor, was dissolved in DMSO at a concentration of 100 μM and stored at –30 ˚C. Monodansylcadaverine (MDC; Sigma-Aldrich), an inhibitor of clathrin endocytosis, was dissolved in DMSO at a concentration of 30 mM and stored at –30 ˚C.

Antiviral activity test

To examine the antiviral activity of acacetin in infected MDCK cells, cells were cultured in monolayer (approximately 2×10^4 cells/mL) in 24-well flat-bottomed plates (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and washed twice with FBS-free MEM. They were diluted to 0.001 MOI using 0.04% BSA-containing FBS-free MEM virus solution (A/PR/8/34, A/Osaka/2024/2009, or A/Osaka/71/2011) and allowed to adsorb for 1 h in a CO_2 incubator at 37 ˚C. The samples were washed once with FBS-free MEM and diluted. They were added to FBS-free DMEM containing 0.4% BSA and 2 μg/mL acetyltrypsin at 500 μL/well of the adjusted sample. After incubation in a CO_2 incubator, the viral supernatant was collected and stored at –80 ˚C. The viral titer of the collected supernatant was measured using a focus-forming reduction assay as previously described by Sakata et al. [22]

As an antiviral reagent, serum-free DMEM (500 μL/well) containing 20 μg/mL acacetin was introduced at different time points: during viral adsorption and entry (-1 to 0 h), and during the replication phase (0–4 h, 4–8 h, or 0–8 h post-infection).

Cell viability was assessed using the method described by Sakata et al. [22]

Time-of-addition assay (viral growth inhibition phase)

To clarify the stage of viral infection and replication at which acacetin exerts its antiviral effects, a time-of-addition assay was performed using MDCK cells and the influenza virus A/PR/8/34, as previously described [22]. MDCK cells seeded in 24-well plates were infected with 0.01 MOI of the influenza A/PR/8/32 virus for 1 h. FBS-free DMEM (500 μL/well) with or without (control) 20 μg/mL acacetin was exchanged at designated time intervals: during adsorption and entry (–1 to 0 h), and during replication (0–4 h, representing the early stage; 4–8 h, representing the late stage; and 0–8 h post-infection, corresponding to one replication cycle). The medium without the sample was supplemented with the same volume of DMSO as that of acacetin. The cells were then cultured for 8 h post-infection and washed with FBS-free MEM. The medium was replaced with a fresh medium, then the infected cells were frozen at –80 ˚C and subjected to two freeze-thaw cycles before determining viral yields using the focus-forming assay.

Influenza virus uptake analysis

To visually demonstrate the inhibitory effect of acacetin on viral entry, influenza virus uptake was analyzed according to the method described by Sakata et al. [22], with slight modifications, Specifically, 20 μg/mL acacetin was used as the inhibitor in place of soybean extract.

Inhibition of viral protein synthesis (western blotting)

To examine the effects of acacetin on viral protein synthesis, the expression levels of viral NP proteins were examined in acacetin-treated cells at each stage of viral replication. Virus-infected

cells were incubated for 8 h according to the time-of-addition assay method, washed with PBS, detached via trypsin treatment, and collected into a 1.5-mL microcentrifuge tubes. The same procedure was repeated twice to resuspend the cells; thereafter, Radio-Immunoprecipitation Assay (RIPA) Buffer (FUJIFILM Wako Pure Chemical Corporation) containing a protease inhibitor cocktail (Roche 04693116001; Roche Life Science, Penzberg, Germany) was added and the cells were kept on ice for 10 min. The sample for western blotting was prepared by adding 4× sample buffer (FUJIFILM Wako Pure Chemical Corporation) to the protein solution and centrifuging at 20,000 × *g* for 10 min at 4 ˚C. The protein solution was then mixed with 4× sample buffer (FUJIFILM Wako Pure Chemical Corporation) and 1 M DTT (final concentration 100 mM), and stored at –30 ˚C. Protein concentrations were measured using the BCA Protein Assay Kit (Takara Bio Inc., Shiga, Japan).

Samples were prepared with sample buffer to a final protein concentration of 10 µg/mL, electrophoresed using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with Block Ace (DS Pharma Biomedical Co. Ltd., Osaka, Japan) for 90 min at 25 ˚C. The viral nucleoprotein (NP) and β-actin were incubated with anti-NP polyclonal antibody (GTX125989, 1:10000 dilution; GeneTex Inc., San Antonio, CA, USA) and anti-β-actin monoclonal antibody (MAB1501, 1:2000 dilution; Chemicon International, Temecula, CA, USA); diluted in Block Ace; and reacted overnight at 4 ˚C. The membranes were washed three times with Tris-buffered saline containing Tween 20 (TBS-T: 0.05M Tris-HCl, 0.15 M NaCl, and 0.05% Tween 20, pH 7.6) for 5 min each. The membranes were then incubated with a Horseradish Peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG, #7074; Cell Signaling Technology, Inc., Danvers, MA, USA) diluted 1:2000 for 1 h at 37 ˚C. The samples were washed three times with TBS-T for 5 min, visualized using an HRP luminescence reagent (ATTO Corporation, Tokyo, Japan), and photographed and observed using an LAS-3000 (FUJIFILM Co. Ltd.).

Examination of vRNA expression (real-time PCR)

To investigate the inhibitory effect of acacetin on vRNA replication and transcription activity, MDCK cells were cultured as a monolayer in 24-well flat-bottomed plates (Thermo Fisher Scientific) and washed twice with FBS-free MEM; thereafter, the viral solution (A/PR/8/34) diluted to 0.1 MOI in FBS-free MEM containing 0.04% BSA was added and adsorbed in an incubator under 5% CO_2 condition at 37 ˚C for 1 h. After washing once with FBS-free MEM, FBS-free DMEM containing 0.4% BSA and 2 µg/mL acetyltrypsin was added to the culture and incubated for 4 h. Real-time PCR was performed using the viral *HA* primer designed for the A/PR8/34 virus as reported by Choi et al. [23] Primers for viral *HA* designed for the A/PR8/34 virus [23], viral *NP* [23], and housekeeping gene *18S ribosomal RNA* (*rRNA*) [24] (Table 1), and the THUNDER BIRD SYBR mixture (TOYOBO Co. Ltd., Osaka, Japan) were used for analysis. Data were normalized to *18S rRNA* and shown as relative quantification values for samples without acacetin.

Primer Name	Primer Sequence
RPS18 Forward	5′-TGAGAAGTTCCAGCACATTTTG-3′
RPS18 Reverse	5′-ATCGATGTCTGCTTTCCTCAAT-3′
HA Forward	5′-TTGCTAAAACCCGGGAGACAC-3′
HA Reverse	5′-CCTGACGTATTTGGGGCACT-3′
NP Forward	5′-GAATGGTGCTCTCTCTGCTTTTGA-3′
NP Reverse	5′-TCCACTTTCCGTTTACTCTCCTG-3′

Table 1: Primer sequences used in real-time PCR.

Endosomal staining in living cells (acridine orange staining)

To investigate the inhibitory effect of acacetin on endosomal acidification, acridine orange staining was performed as described by Sakata et al. [22], a modification of the original method by Garozzo et al. [25] MDCK cells were cultured as monolayers in 24-well flat-bottom plates (Thermo Fisher Scientific) and rinsed twice with FBS-free MEM. MEM containing acacetin (20 µg/mL) or 100 nM bafilomycin A1 (Funakoshi: V-ATPase inhibitor) was added and incubated in a CO2 incubator for 1 h. Acridine orange (2.5 µg/mL in PBS) was added and stained at 37 °C for 10 min in the dark. After the cells were washed, the cells were exposed to FBS-free and phenol red-free MEM containing 0.04% BSA and observed under a fluorescence microscope with a BZ-X GFP filter (OP-87763) (BZ-X800; KEYENCE, Osaka, Japan). Acridine orange changed from green to red with decreasing pH.

Early endosomal and lysosomal staining in living cells

To investigate the inhibitory effect of acacetin on the transformation of early endosomes into late endosomes, staining was performed according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan), with minor modifications. HeLa cells were cultured as a monolayer in 35-mm glass bottom dishes and washed once with PBS. A viral solution (A/PR/8/34) diluted to an MOI of 10 using 0.04% BSA-containing FBS-free MEM was prepared. Acacetin (20 µg/mL) and the same amount of DMSO were diluted in virus-containing FBS-free MEM, added at 1 mL/dish, and incubated in a CO_2 incubator at 37 ˚C for 30 min. After incubation, ECGreen-Endocytosis Detection (Dojindo Laboratories Co., Ltd., Kumamoto, Japan), diluted 1000-fold using MEM-medium containing 0.04% BSA, was added at 1 mL/dish and incubated

in a CO_2 incubator at 37 ˚C for 90 min in the dark. LysoTracker™ Red DND-99 (Thermo Fisher Scientific) diluted to 0.25 mM with DMSO was added directly to obtain a 2000-fold dilution 30 min before the end of incubation. The cells were then washed twice with PBS. FBS-free 2 × MEM containing 0.04% BSA was added, and the cells were observed under a fluorescence microscope (KEYENCE BZ-X800).

Analysis of transferrin and cholera toxin B (CTxB) uptake

To investigate the inhibitory effect of acacetin on the clathrin- and caveolae-dependent endocytic pathways, the cellular uptake of transferrin and CTxB, internalized via the clathrin- and caveolae-dependent endocytic pathway, respectively, was observed in HeLa cells through fluorescence microscopy (KEYENCE BZ-X800) [22].

Fluorescence intensity was measured by analyzing fluorescence photographs using ImageJ (version 1.53g, 4 December 2020: https://imagej.nih.gov/ij/). The Region of Interest (ROI) was defined based on the cell morphology, and the average fluorescence intensity of the ROIs from 30 cells was calculated.

Statistics

The amounts of virus during antiviral assays were analyzed using a two-tailed Student's *t*-test in Excel Toukei (version 6.0; Esumi, Tokyo, Japan). Values are presented as mean ± standard deviation. A p-value < 0.05 was considered statistically significant.

Results

Anti-influenza virus activity of acacetin

In a cell viability test of acacetin against MDCK cells, no cytotoxicity was observed at concentrations below 28.4 µg/mL; therefore, the maximum concentration of acacetin added in the antiviral activity test was set at 28.4 µg/mL. The anti-influenza viral activity of acacetin was examined using influenza A virus H1N1 A/PR/8/34 and two oseltamivir-resistant viruses, Osaka/2024/2009 and Osaka/71/2011, and compared with that of the PR8 strain (Table 2). The antiviral activities of acacetin against the two Osaka strains ($p = 0.008$, $p = 0.001$) were significantly lower than its activity against the PR8 strain.

Virus	Antiviral activity
	IC_{50} (µg/mL)
PR/8/34	5.0 ± 2.0
Osaka/2024/2009*	16.7 ± 0.4
Osaka/71/2011*	18.1 ± 1.6

*Oseltamivir-resistant viruses

IC_{50} (the concentration of acacetin that inhibits viral growth by 50% compared to the viral titer of a sample cultured in acacetin-containing medium).

Table 2: Antiviral activity of acacetin against influenza A virus H1N1.

Acacetin was added to infected cells at designated time intervals as a time-of-addition assay to determine the growth stage at which IFV inhibition occurred. Based on virus titer levels, acacetin inhibited all stages of the entry phase, and early and late cultivation stages (Figure 1).

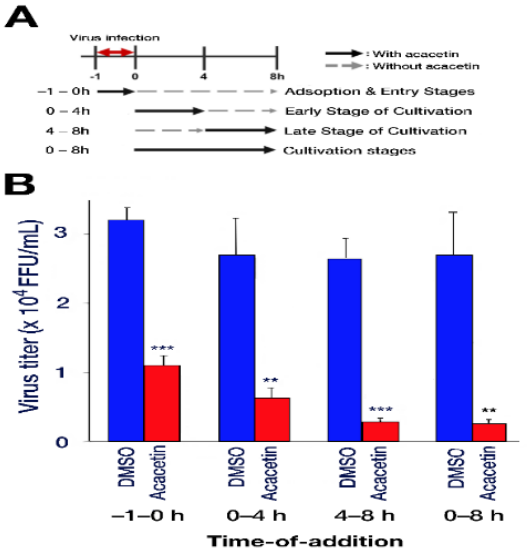

Figure 1: Determination of the viral growth phase inhibited by acacetin.

A: Schedule of time-of-addition assay. MDCK cells were infected with influenza virus (PR/8/34), and medium containing acacetin was added at –1 to 0 h (sample added only at adsorption and entry), 0 to 4 h (sample added only at the early stage of culture), 4 to 8 h (sample added only at the late stage of culture), as well as 0 to 8 h (corresponding to one replication cycle). B: The virus titer was determined by antiviral activity test, after incubation. MDCK, Madin–Darby canine kidney

Effect of acacetin activity on virus uptake

It is known that the influenza virus infects both MDCK cells and HeLa cells [22]; however, this study was conducted to determine whether the same phenomenon occurs in HeLa cells, which are of human origin, as it does in MDCK cells, which are of canine origin. As acacetin inhibits the entry phase in both cells, we stained the viral NP protein to observe its effect on viral behavior after infection. Fluorescence microscopy showed the localization of the virus on the plasma membrane in acacetin-treated cells, whereas in control cells, the cytoplasm and nuclei were stained, confirming that the virus was taken up into the cells. This indicated that acacetin inhibits viral entry into the cell and endocytosis (Figure 2). The results suggest that differences in cell type are unlikely to influence the virus's entry mechanism.

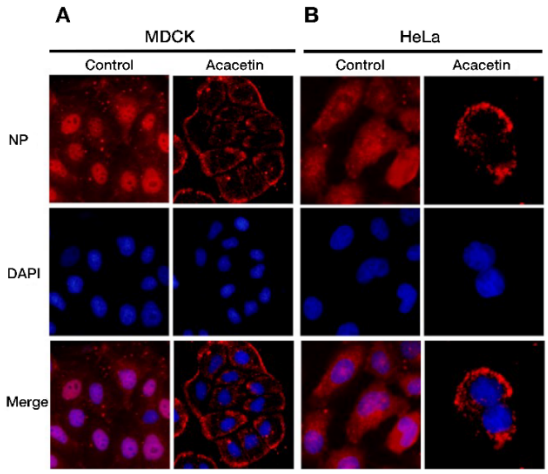

Figure 2: Localization of influenza virus NPs.

MDCK and HeLa cells were infected with influenza A/PR/8/34 virus. Fluorescence microscopy was used for observing: **A**, MDCK and **B**, HeLa cells.

Red: influenza virus NPs; blue: DAPI-stained cell nuclei. MDCK, Madin–Darby canine kidney

Effect of acacetin on viral protein synthesis and RNA expression levels

Acacetin inhibits the early stage of viral growth; therefore, we investigated the regulation of viral protein expression. The viral growth stage inhibited by acacetin was examined using a time-of-addition assay and western blotting (Figure 3A). The expression of the viral NP protein decreased when acacetin was applied for 0 to 4 h.

To investigate the detailed inhibition mechanism of acacetin in the early stage of viral growth, we examined the RNA expression levels of the viral proteins, *HA* and *NP*, using real-time PCR. The addition of acacetin significantly decreased the RNA expression levels of *HA* ($p = 0.003$) and *NP* ($p = 0.020$) (Figure 3B).

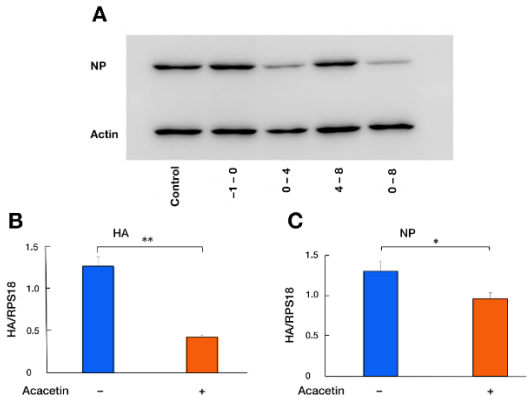

Figure 3: Change in viral RNA expression by the addition of acacetin.

A: MDCK cells were infected with influenza virus and treated with acacetin as in Figure 1. After incubation for 8 h, the proteins were collected, and the expression levels of NPs and β-actin were examined using SDS-PAGE and western blotting.

B: MDCK cells were infected with influenza virus (PR/8/34); thereafter, medium containing acacetin was added and the cells were allowed to grow for 4 h. After RNA extraction, cDNA was synthesized and quantified using real-time PCR. The *HA* and *NP* RNA expression levels per *RPS18* RNA expression level were examined. *HA*/*RPS18* levels without or with acacetin were 1.27 ± 0.11 and 0.43 ± 0.10; *NP*/*RPS18* levels without or with acacetin were 1.30 ± 0.11 and 0.94 ± 0.07 (n = 5), respectively. The data are representative of three independent experiments. (*$p < 0.05$, **$p < 0.01$). The statistical test Student's *t*-test in Excel was used. MDCK, Madin–Darby canine kidney

Effect of acacetin on endosomal acidification

The effect of acacetin on endosomal acidification was examined using acridine orange and ECGreen/LysoTracker staining. Red staining was not observed for the proton ATPase inhibitor, Bafilomycin A1, whereas red staining was observed for acacetin and the control, indicating endosome acidification (Figure 4A).

To confirm the results of endosome acidification by acridine orange staining, we used ECGreen/LysoTracker to stain endosomes and lysosomes, and examined their effects on endosome migration

(Figure 4B). Endosomes become late endosomes and transitioned to lysosomes upon acidification. The ratio of yellow staining denoting colocalization of endosomes and lysosomes to green staining denoting endosomes was not significantly different between the control and acacetin groups ($p = 0.083$) (Figure 4C).

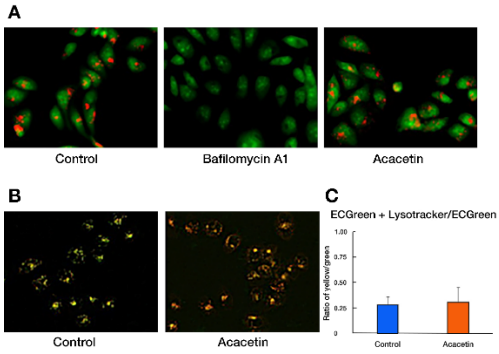

Figure 4: Effect of acacetin on endosomal acidification.

A: MDCK cells were incubated with acacetin and Bafilomycin A1 in medium with or without Bafilomycin A1. The cells were observed using a fluorescence microscope (KEYENCE BZ-X800).

B: HeLa cells were incubated with a viral solution (A/PR/8/34) and with or without acacetin. After incubation, ECGreen-Endocytosis Detection treatment was performed according to the manufacturer's instructions, and LysoTracker™ Red DND-99 was added 30 min before the end of incubation. After incubation, 2 × MEM was added and the cells were observed under a fluorescence microscope.

C: The number of cells and green and yellow areas per cell were calculated using image analysis software (KEYENCE BZ-X800 Analyzer). The yellow/green area ratio of 20 cells extracted using Excel Random function is shown in the graph and the ratio without or with acacetin were 0.282 ± 0.077 and 0.208 ± 0.137, respectively. MDCK, Madin–Darby canine kidney; MEM, minimum essential medium

Effect of acacetin on transferrin and CTxB uptakes

Acacetin inhibits endocytosis, therefore, we examined it effect on clathrin-dependent endocytosis. Transferrin uptake into cells was observed using fluorescence microscopy and quantified using image analysis, which showed that acacetin inhibits transferrin uptake in HeLa cells (Figure 5A). This indicates that acacetin affects clathrin-dependent endocytosis and significantly inhibits viral cellular uptake ($p = 0.008$) (Figure 5B).

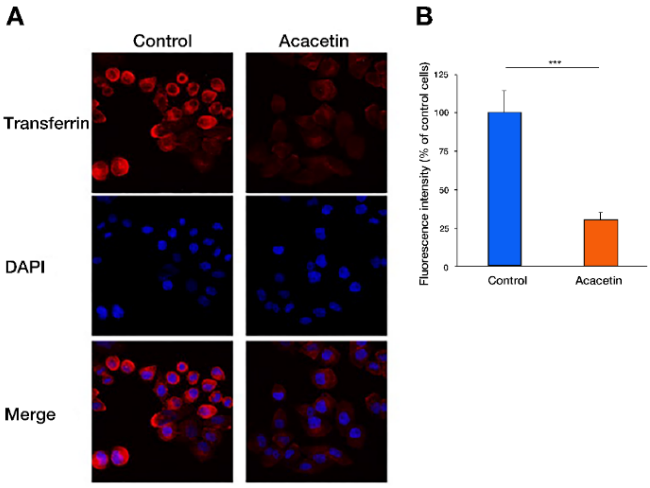

Figure 5: Effect of acacetin on transferrin endocytosis.

A: HeLa cells were incubated with Texas Red-conjugated transferrin-containing medium. After incubation for incorporation into the cells, cells were fixed and observed under a fluorescence microscope. **B**: Fluorescence intensity per cell in **A** was quantified (the intensity of control cells with acacetin: 30.33 ± 5.12 of 30 cells each; ***$p < 0.001$). The statistical test Student's *t*-test in Excel was used. DAPI, 4′,6-diamidino-2-phenylindole

Next, we examined whether acacetin affects caveolae-dependent endocytosis. CTxB uptake by HeLa cells was observed using fluorescence microscopy and quantified using image analysis, which showed no difference in uptake with the addition of acacetin (Figure 6A). This indicates that acacetin does not affect caveolae-dependent endocytosis (Figure 6B).

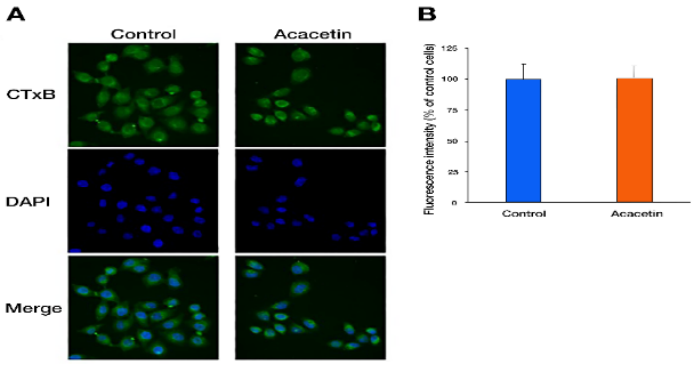

Figure 6: Effect of acacetin on CTxB endocytosis.

A: HeLa cells were incubated with Alexa Fluor 488-conjugated CTxB-containing medium and with or without acacetin. After incubation for incorporation into the cells, cells were fixed and observed under a fluorescence microscope. **B**: Fluorescence intensity per each cell in **A** was quantified (the intensity of control cells with acacetin: 100.00 ± 0.10 of 30 cells each). The statistical test Student's *t*-test in Excel was used. CTxB, cholera toxin B; *DAPI*, 4′,6-diamidino-2-phenylindole

Effect of acacetin on clathrin cellular localization

To investigate the mechanism of the clathrin-dependent inhibition of endocytosis by acacetin, we examined the localization of clathrin during the adsorption phase of the viral growth. We observed clathrin accumulation in the Golgi apparatus upon the addition of MDC and acacetin, which may be linked to clathrin-dependent endocytosis (Figure 7).

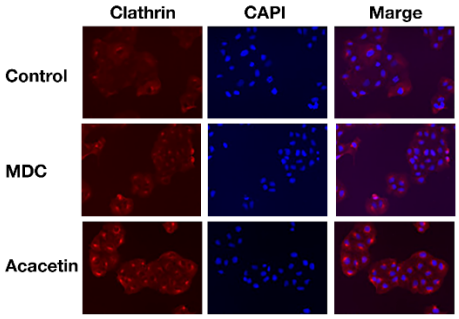

Figure 7: Clathrin fluorescence immunostaining.

MDCK cells were treated with virus (A/PR/8/34) and acacetin, and samples with or without MDC were added. The cells after incubation were fixed with paraformaldehyde, fixed by acetone-methanol, and permeabilized with Trion-X. After blocking with BSA, primary antibody (anti-clathrin heavy chain), secondary antibody (goat anti-mouse Alexa flour 594), and DAPI were reacted. After adding the mounting agent and fixing the cells onto glass slides, the slides were observed under a fluorescence microscope. Red: clathrin; blue: nuclei. *DAPI*, 4′,6-diamidino-2-phenylindole; MDC, monodansylcadaverine; MDCK, Madin–Darby canine kidney

The overall results of this study indicate that acacetin inhibits viral uptake into cells via clathrin-dependent endocytosis by clathrin accumulation near the Golgi apparatus. Furthermore, acacetin was shown to inhibit vRNA replication and transcription.

Discussion

In this study, the mechanism underlying the anti-influenza viral action of acacetin was elucidated. Acacetin inhibited viral growth during all stages, including adsorption, early culture, and late culture, entry into host cells by inhibiting clathrin-dependent endocytosis.

We investigated the inhibitory mechanism during late viral growth in culture and found that the antiviral activity of acacetin against the two oseltamivir-resistant Osaka strains was lower than that against the oseltamivir-sensitive PR8 strain (Table 2). The time-of-addition assay indicated that acacetin inhibited all stages of virus replication; virus entry, early (vRNA synthesis), and late (protein synthesis and virus maturation) stages (Figure 1). These suggest that the antiviral effects of acacetin on late-stage cultures may be attributed to NA inhibition. NA cleaves the viral envelope at sialic acid residues during viral budding [25]. NA inhibitors exhibit antiviral activity by competitively binding to the active site of NA [26-28]. The flavonoid, 8-prenylnaringenin (8-PN), which has been studied at our laboratory, is a phytoestrogen and has low antiviral activity against oseltamivir-resistant strains [21]. However, it is unclear whether 8-PN directly inhibits NA activity in a manner similar to acacetin, which should be explored in future research. Further investigation of the inhibitory activity of acacetin against the Osaka strain at the late culture stage and measurement of NA activity are needed to elucidate the mechanism of antiviral action at the late culture stage in more detail.

We investigated the effect of acacetin on viral protein synthesis. During the early stages of viral replication, when vRNA transcription and replication primarily occur, acacetin treatment reduced NP expression (Figure 3A). NP expression did not decrease after acacetin treatment in the late stages (Figure 3A). Real-time PCR showed that acacetin reduced the vRNA expression (Figure 3B). Therefore, acacetin exhibits antiviral activity during the early stages of culture by inhibiting vRNA synthesis, similar to daidzein. Acacetin may inhibit RNA polymerase, which is responsible for vRNA replication and the synthesis of messenger RNA via transcription. However, it remains unclear whether acacetin's inhibition of RNA synthesis results from direct or indirect inhibition of RNA polymerase activity, and whether its mechanism of action is the same as that of daidzein; these questions should be elucidated in future studies.

Staining with acridine orange and ECGreen/LysoTracker showed that acacetin does not inhibit endosomal acidification. Fluorescent immunostaining of clathrin showed the accumulation of clathrin in the Golgi apparatus upon the addition of acacetin. Clathrin is translocated to the plasma membrane by various adaptor and accessory proteins, including adaptor protein 2 and Epsin1, to form clathrin capsular vesicles [29]. Once clathrin capsular vesicles are excised by dynamin and incorporated into the cell, they undergo deshelling as they pass from early to late endosomes and fuse with lysosomes, and virus ribonucleoproteins migrate into the nucleus [30,31]. During this process, clathrin capsular vesicles are released from endosomes and some are translocated to recycling endosomes near the Golgi for recycling [29]. Furthermore, it has been suggested that this phenomenon of claslin

accumulation near the Golgi apparatus results from the inhibition of claslin recycling [32]. Based on these observations, we suggest that clathrin accumulation in the Golgi is due to the inhibition of clathrin transport by recycling endosomes to the plasma membrane by acacetin. Further, we speculated that clathrin-dependent endocytosis inhibits viral entry. ACAP1 and AP1B are adapter proteins for recycling endosomes to the plasma membrane [33-35]. Further studies on the expression and functional effects of acacetin on these proteins are required to elucidate the mechanism underlying the inhibition of clathrin-dependent endocytosis.

Acacetin, is a natural flavonoid compound that possesses anticancer, anti-inflammatory [36], and antioxidant [37] activities. Acacetin inhibits the PI3K/Akt signaling pathway [38]. The PI3K signaling pathway is an intracellular signaling pathway that is activated by extracellular stimuli and also responds to influenza virus infection [39-41], and its activation promotes viral endocytosis [42]. The inhibition of the Ras/PI3K signaling pathway inhibits viral infection via clathrin-independent endocytosis [42] and PI3K/Akt signaling plays a regulatory role in multiple endocytosis pathways [43]. Thus, acacetin may inhibit the cellular uptake of the influenza virus by inhibiting the PI3K/Akt signaling pathway and affecting endocytosis.

Although our study suggests that acacetin inhibits influenza virus entry by inhibiting clathrin-dependent endocytosis, we did not elucidate the mechanism by which acacetin inhibits intracellular signaling pathways required for clathrin-dependent endocytosis progression as the intracellular signaling pathways from acacetin are not yet known. Further studies are required to elucidate the effects of acacetin on the intracellular signaling pathways and proteins required for the progression of clathrin-dependent endocytosis.

Conclusion

Acacetin, obtained from soybean extract, inhibits influenza virus activity during the viral invasion phase. In this study, acacetin inhibited viral entry by inhibiting clathrin-dependent endocytosis. Acacetin is speculated to directly or indirectly inhibit RNA polymerase, which is responsible for vRNA replication and transcription. Thus, acacetin is a potential antiviral drug candidate that has effects that are distinct from those of existing drugs. Further biochemical and molecular biological studies are required to elucidate the mechanism by which acacetin inhibits clathrin-dependent endocytosis and vRNA polymerase.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author Contributions

Moe Ishinaka and **Natsumi Sakata**: Formal analysis, data curation, and investigation. **Yuka Horio**: Methodology, formal analysis, and supervision. **Yoshihisa Nakano**: supervision. **Yuji Isegawa**: Funding acquisition, conceptualization, project administration, and writing – original draft, review, and editing.

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