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Original Article

Rosmarinic acid interferes with influenza virus A entry and replication by decreasing GSK3β and phosphorylated AKT expression levels

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Akt;
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Abstract *Background:* The purpose of this study was to examine the *in vivo* activity of rosmarinic acid (RA) — a phytochemical with antioxidant, anti-inflammatory, and antiviral properties — against influenza virus (IAV). An antibody-based kinase array and different *in vitro* functional assays were also applied to identify the mechanistic underpinnings by which RA may exert its anti-IAV activity.

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Rosmarinic acid;
Signaling pathways

Methods: We initially examined the potential efficacy of RA using an *in vivo* mouse model. A time-of-addition assay and an antibody-based kinase array were subsequently applied to investigate mechanism-of-action targets for RA. The hemagglutination inhibition assay, neuraminidase inhibition assay, and cellular entry assay were also performed.
Results: RA increased survival and prevented body weight loss in IAV-infected mice. *In vitro* experiments revealed that RA inhibited different IAV viruses — including oseltamivir-resistant strains. From a mechanistic point of view, RA downregulated the GSK3β and Akt signaling pathways — which are known to facilitate IAV entry and replication into host cells.
Conclusions: RA has promising preclinical efficacy against IAV, primarily by interfering with the GSK3β and Akt signaling pathways.

Introduction

Influenza viruses can infect a wide range of host species and are mainly transmitted through respiratory droplets. Taxonomically, they can be divided into four types (A, B, C, and D) and further into subtypes based on the antigen nature of viral membrane glycoproteins.[1] The genome of influenza A virus (IAV) comprises eight negative-sense, single-stranded viral RNA segments which encode at least 10 proteins[2] — including hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 (M1) and 2 (M2), polymerase acidic (PA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), nucleoprotein (NP), and non-structural proteins 1 (NS1) and 2 (NS2). HA and NA are the most abundant viral surface glycoproteins. Eighteen subtypes of IAV HA (H1–H18) and 11 subtypes of IAV NA (N1–N11) have been identified to date.[2] IAV infections are initiated by attachment of HA to host cell surface receptors, including *sialic acid*-containing glycans.[3,4] Sialic acid is subsequently cleaved by NA to promote the release of progeny virions.[5] Additionally, NA can facilitate virus entry.[6,7]

While vaccination is a highly effective method of preventing IAV, the cross-protection against different subtypes is still limited. Moreover, cross-protection induced by vaccines during influenza epidemics remains unpredictable. Flu antiviral drugs are clinically useful to treat seasonal influenza outbreaks. In general, anti-influenza drugs may be classified according to their viral targets (i.e., PA, M2, and NA). Baloxavir marboxil (Xofluza) inhibits viral replication by targeting the PA activity required for cap-snatching.[8,9] Amantadine and rimantadine are M2 proton channel blockers that inhibit influenza virus genome trafficking to the host cell nucleus.[10,11] Oseltamivir (Tamiflu) and zanamivir (Relenza) are NA inhibitors that prevent the release of virus progeny. From a mechanistic point of view, these drugs inhibit removal of sialic acid moieties from glycans linked to HA.

Unfortunately, prolonged treatment may induce the emergence of resistant mutants.[12] In this regard, Bright et al.[13] have previously shown that adamantane resistance in IAV isolates increased from <10% in 2000–2002 to 57.5% and 73.8% in 2003 and 2004, respectively. Neuraminidase inhibitor resistance has also emerged in 2007–2008 as a result of viruses carrying a H274Y NA mutation.[14] A I38X substitution within the PA endonuclease domain has also been observed in 9.7% of patients treated with baloxavir marboxil.[15] Collectively, these data indicate that there is still an unmet need for new and effective treatment options against influenza.

Rosmarinic acid (RA) is a phytochemical with broad-spectrum antiviral activities. For example, RA can directly block the function of the human immunodeficiency virus-1 enzyme integrase.[16] Moreover, RA inhibits hepatitis B virus replication by targeting the viral epsilon RNA-polymerase interaction.[17] Finally, we have recently shown that RA directly inhibits the interaction between enterovirus A71 virions and their host receptors.[18] The purpose of this study was to examine the *in vivo* activity of RA against IAV infections. An antibody-based kinase array and different *in vitro* functional assays were also applied to identify the mechanistic underpinnings by which RA may exert its anti-IAV activity.

Methods

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were cultured in E_{10} medium consisting of Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD, USA), 0.1 mM nonessential amino acid (NEAA) mixture (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma–Aldrich, St Louis, MO, USA). Human lung carcinoma A549 cells were cultured in minimal essential medium (MEM; Invitrogen) containing 10% heat-inactivated FBS and penicillin/streptomycin. Human embryonic kidney (HEK) 293 cells were maintained in DMEM containing 10% FBS. All cell lines were grown at 37 °C under a humidified 5% CO_2 atmosphere. The influenza virus A/WSN/33 (WSN) was obtained from the American Type Culture Collection (Manassas, VA, USA) and propagated in MDCK cells. The sources and proliferation conditions for other viruses — including H1N1pdm09,[19] human herpes

simplex virus type 1 (HSV-1), and adenoviruses — have been previously described in detail.[20] Viral titers were determined with a conventional plaque assay.[21]

Animal experiments

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Chang Gung University (approval number: CGU10-001). Four-week-old SPF BALB/c mice were obtained from Lasco (Taipei, Taiwan). RA (100 μg dissolved in 100 μL of 5% dimethyl sulfoxide [DMSO] aqueous solution) was administered orally twice a day (100 mg/kg per day). An equal volume of 5% DMSO was used as negative control. Once the mice (n = 10 per group) were properly anesthetized, an intranasal challenge with influenza virus A/WSN/33 (2 × median lethal dose, LD_{50}, 2 × 10^4 plaque-forming units [PFU] in 20 μL) was performed. All infected mice were monitored daily for body weight and survival. Weight tracking was halted when a change in group number occurred. Mice were considered dead if weight loss surpassed 25%. In cytokine quantification experiments, mice from each group were euthanized via CO_2 inhalation five days after infection. Harvested lung tissues were weighed and homogenized in ice-cold PBS. Lung homogenates were centrifuged at 300 *g* for 10 min at 4 °C. The supernatants were stored in aliquots and subsequently used for quantifying tumor necrosis factor (TNF)-α levels with the mouse ELISPOT ReadySETGo! ELISA kit (eBioscience, San Diego, CA, USA).

Inhibition of virus-induced cell death (anti-cytopathic effect) assay and cytotoxicity assessment

MDCK cells (1.5 × 10^4 cells/well) were seeded in 96-well tissue culture plates and infected with 9 × 50 percent tissue culture infective dose (9TCID50) of different influenza virus strains in presence of different RA concentrations (Sigma—Aldrich). Cells were maintained in E_0 (DMEM containing penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM NEAA mixture, and 2.5 μg/mL trypsin). The half-maximal cytotoxic concentration (CC_{50}) and half-maximal inhibitory concentration (EC_{50}) were determined as previously described.[21]

Plaque reduction assay

MDCK cells (5 × 10^5 cells/well) were seeded into 6-well tissue culture plates and incubated overnight. Cell monolayers were infected with influenza virus A/WSN/33 (~45 PFU/well) either in the absence or presence of different RA concentrations. Following 1 h of adsorption at 37 °C, the viral suspension was removed and cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were treated with E_0 containing 0.3% agarose in presence of different RA concentrations. The antiviral activity of RA was analyzed with a plaque reduction assay with respect to the negative control (DMSO). The ImageJ software and the ViralPlaque macro were used for plaque size quantification.[22]

Time-of-addition assay

A 6-well tissue culture plate was seeded with MDCK cells (5 × 10^5 cells/well) and incubated at 37 °C for 16—20 h under a 5% CO_2 atmosphere. Cells were infected with influenza virus A/WSN/33 at the indicated multiplicity of infection (MOI) for 1 h and kept on ice for 1 h. Following viral adsorption, cells were washed with Hank's balanced salt solution (HBSS) to remove unbound viruses. Different concentrations of RA dissolved in E_0 were added at the time points reported in Fig. 3. Supernatants were harvested at 12 h post infection (hpi) and viral titers were determined using the plaque assay.[21]

RNA extraction and quantitative reverse transcription-polymerase chain reaction

MDCK cells (5 × 10^5 cells/well) were seeded into 6-well tissue culture plates, infected with either influenza virus A/WSN/33 or A/90206 (MOI: 0.01) for 1 h, and subsequently treated with RA. Cells were harvested at 0, 6, 9, and 12 hpi. Total intracellular RNA was extracted with the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA levels of influenza virus A/WSN/33 or A/90206 were measured by quantifying M1 and NA expression, respectively. The following primers were used for real-time PCR: M1 forward 5′-GAC CAA TCC TGT CAC CTC-3′ and M1 reverse 5′-GAT CTC CGT TCC CAT TAA GAG-3′; NA forward 5′-CAC CAA CTT TGC TGC TGG ACA-3′ and NA reverse 5′-TGA CAA ACA CAT CCC CCT TGG-3′; glyceraldehyde phosphate dehydrogenase (GAPDH) forward 5′-AAG AAG GTG GTG AAG CAG GC-3′ and GAPDH reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Hemagglutination and hemagglutination-inhibition assays

Following two-fold serial dilution in PBS, influenza virus A/WSN/33 was mixed for 1 h with two volumes of guinea pig red blood cells (RBCs) in round-bottomed 96-well plates. RBC aggregation caused by the lowest viral titer was defined as 1 × hemagglutination value (HAv).[23] A virus titer of 4 × HAv was used for the hemagglutination-inhibition (HAI) assay, in which different RA concentrations were mixed with influenza virus A/WSN/33 at room temperature for 30 min. Subsequently, the mixture was incubated for 1 h with two volumes of guinea pig RBCs.

RdRp activity assay

HEK293 cells (5 × 10^4 cells) were seeded in a 48-well plate — which was precoated with poly-L-lysine for 30 min — and incubated at 37 °C for 16—20 h. Subsequently, cells were cotransfected with 0.1 μg of pHW2000-NP, -PA, -PB1, and -PB2, as well as with pPOLI-FLuc-RT — a plasmid encoding firefly luciferase driven by POLI (0.1 μg) — and pRL-TK —

which carried Renilla luciferase as an internal control (5 ng). After 8 h of incubation, the transfection complex was replaced with growth medium supplemented with the reported RA concentrations. The ribonucleoprotein (RNP) complex activity was assayed after 48 h using a Dual-Luciferase Assay System (Promega, Madison, WI, USA).

Antibody-based detection of protein phosphorylation status

A 6-well tissue culture plate was seeded with A549 cells (5×10^5 cells/well) and incubated at 37 °C for 16—20 h under a 5% CO_2 atmosphere. Cells were infected with the

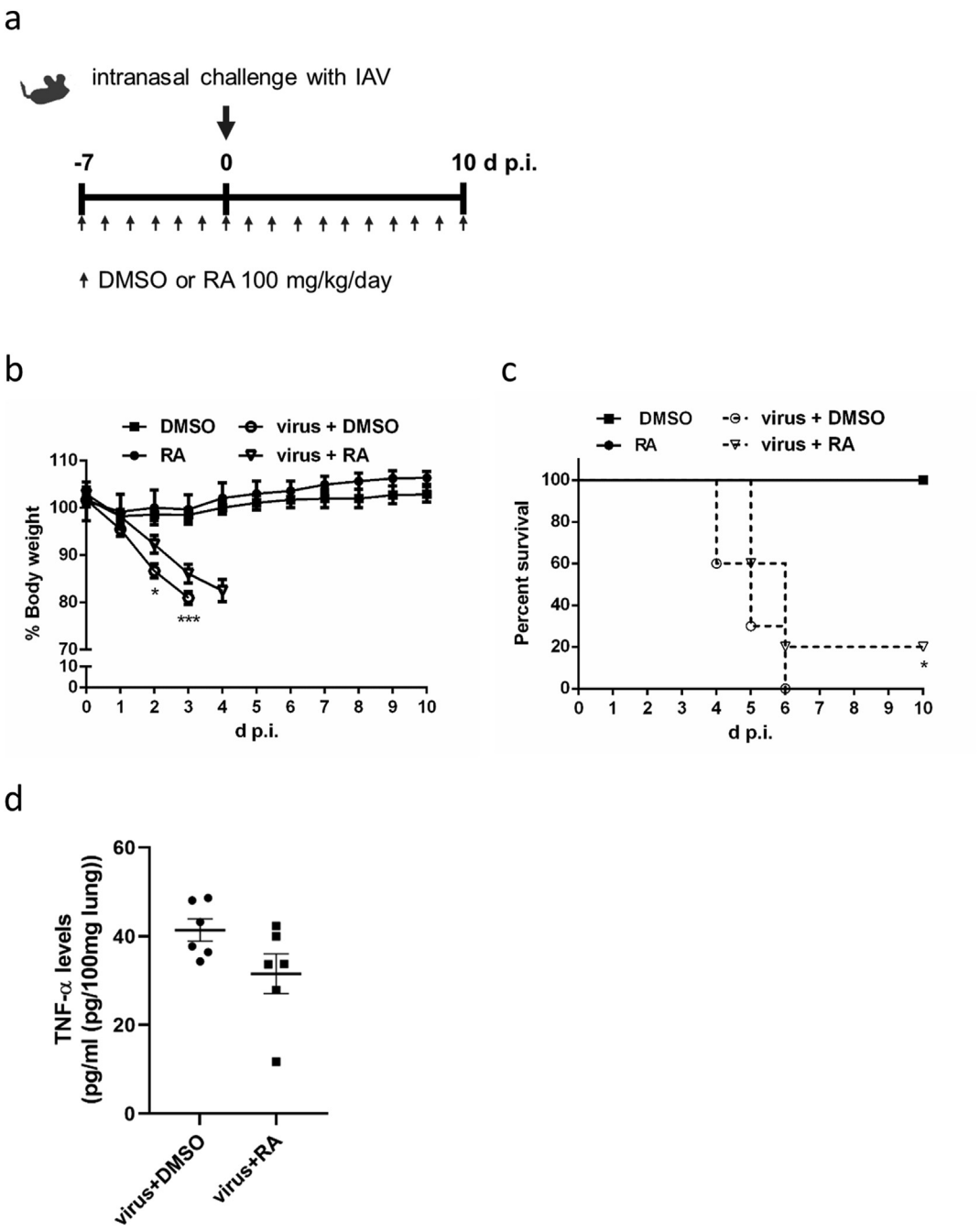

Figure 1. Effects of rosmarinic acid in mice experimentally infected with influenza virus A/WSN/33. (a) Four-week-old BALB/c mice (n = 10 per group) were given either DMSO 5% (100 μL) or rosmarinic acid (100 mg/kg daily) for seven days before an intranasal challenge with influenza virus A/WSN/33. Treatment was subsequently continued for additional ten days after infection. Body weight was measured daily and normalized to the average weight on day 0. Mice were considered dead if weight loss surpassed 25%. (b) Body weight is expressed as the mean ± standard error of the mean. (c) Kaplan—Meier survival plots of mice. *$P < 0.05$ and ***$P < 0.001$. (d) TNF-α levels in lung homogenates from control and RA-treated mice. Values are means ± standard errors of the mean. $P = 0.06$ *versus* control mice.

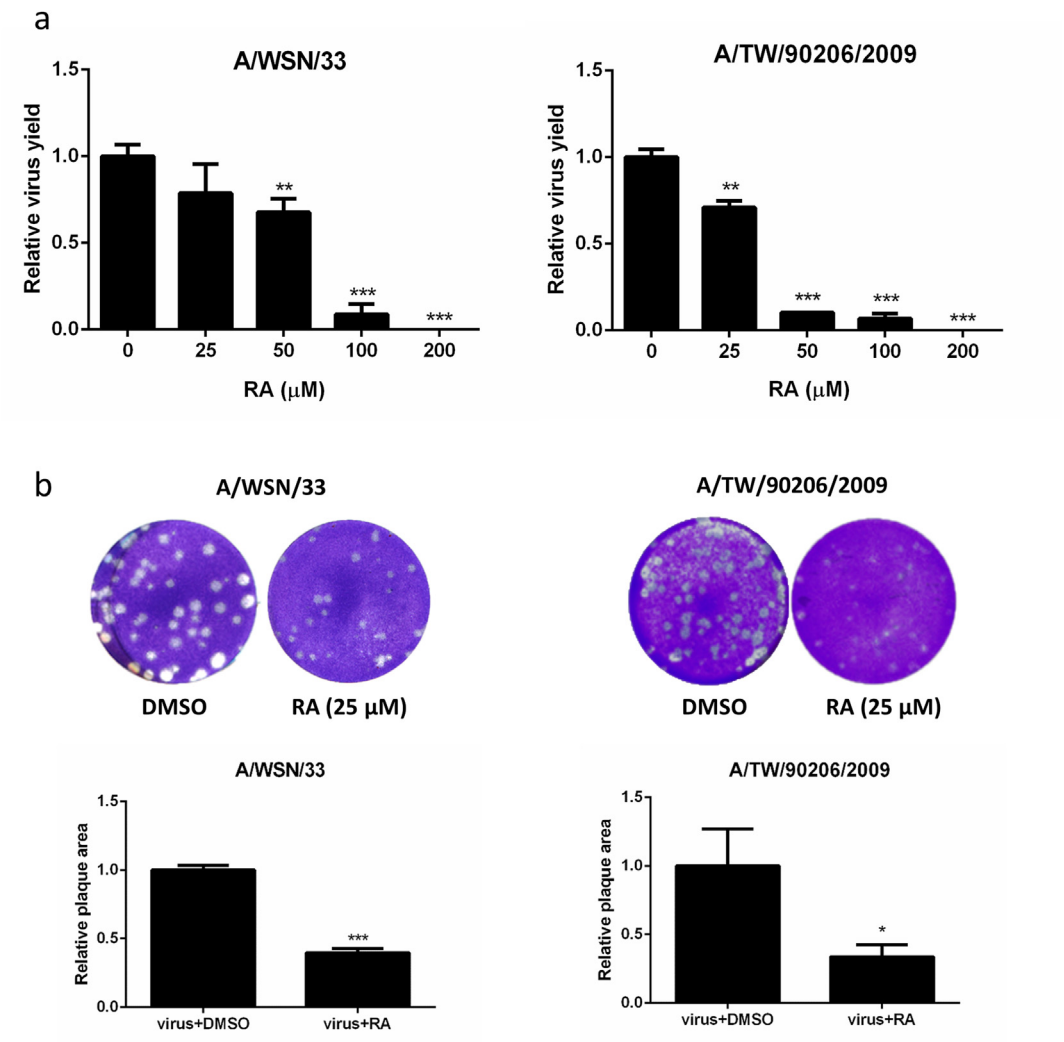

Figure 2. Effects of rosmarinic acid as determined by the plaque reduction assay. (a) Cell monolayers were infected with influenza virus A/WSN/33 (~45 plaque-forming units/well) in absence or presence of different rosmarinic acid concentrations. Following 1 h of adsorption at 37 °C, the viral suspension was removed and cells were washed with phosphate-buffered saline. Subsequently, cells were treated with E0 containing 0.3% agarose in presence of different rosmarinic acid concentrations. The antiviral activity of rosmarinic acid was analyzed on the third post-infection day with a plaque reduction assay; DMSO was used as negative control (arbitrarily set at 1). (b) Plaque size quantification in presence or absence of rosmarinic acid (25 μM). The ImageJ software and the ViralPlaque macro were used for plaque size quantification.[22] Data are means ± standard deviations from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

influenza virus A/WSN/33 (MOI: 0.01) at 37 °C for 1 h and subsequently washed twice with HBSS. Following the addition of RA (100 μM), cells were harvested at 24 hpi and analyzed using a human phospho-kinase array (R&D systems, Minneapolis, MN, USA).

Antibodies for western blot analysis

The following antibodies were used for western blot analysis: mouse anti-phospho-AKT (Ser473) antibody (Cell Signaling, Beverly, MA, USA), rabbit anti-AKT antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho-PRAS40 antibody (GeneTex, Irvine, CA, USA), rabbit anti-PRAS40 antibody (GeneTex), rabbit anti-phospho-GSK3β (Ser9) antibody (Cell Signaling), and rabbit anti-GSK3β antibody (Cell Signaling). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control and was detected using a rabbit anti-GAPDH antibody (Santa Cruz Biotechnology).

Data analysis

The mice survival curves were plotted with the Kaplan–Meier method and compared using log-rank test. The results of *in vitro* experiments – i.e., plaque reduction assays, time of addition assays, western blot analyses, and assays to test the effect of RA on viral RNA synthesis and RdRp complex activity – were analyzed with the Student's *t*-test. Two-tailed P values < 0.05 were considered statistically significant.

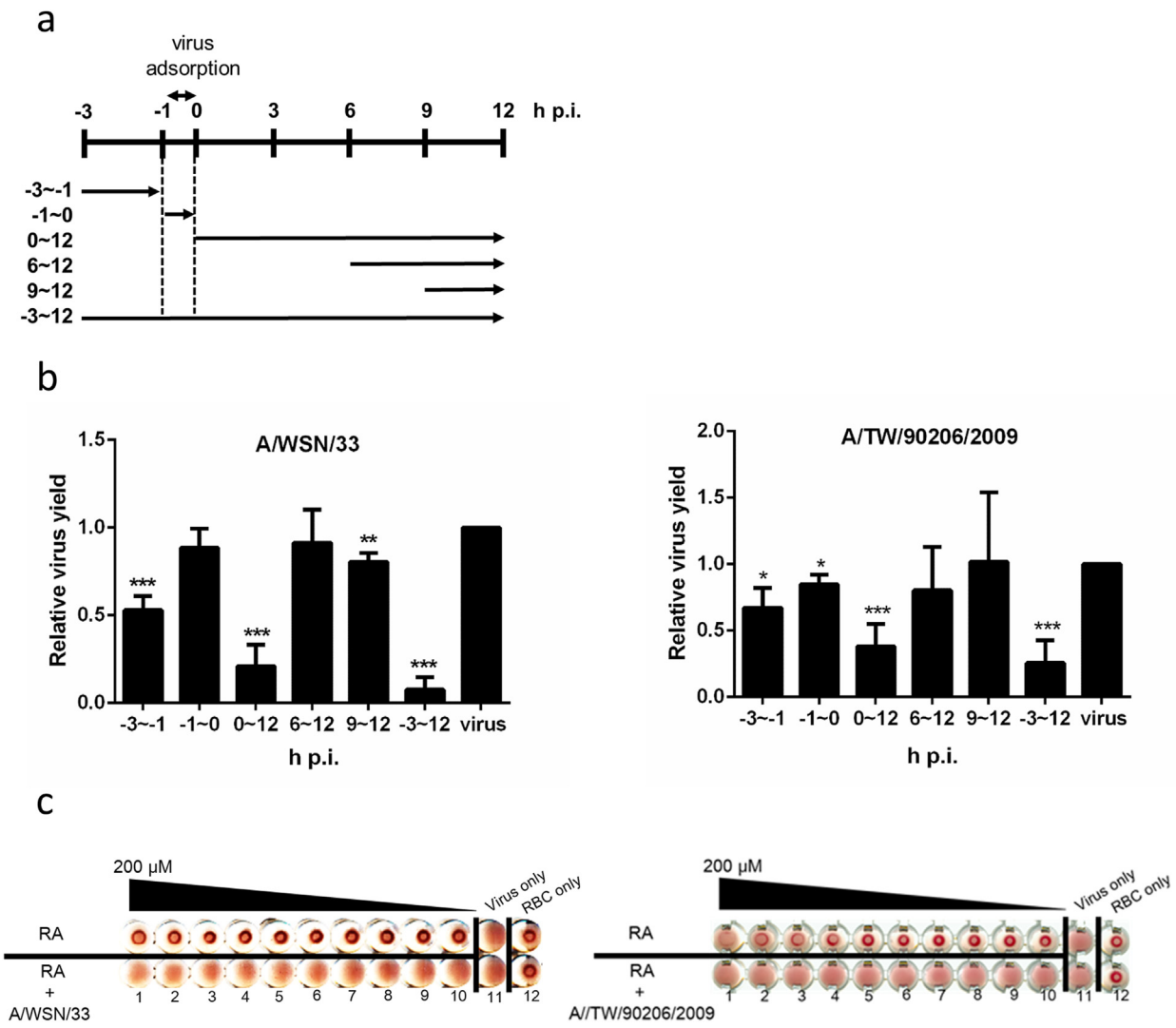

Figure 3. Mechanistic underpinnings of rosmarinic acid against influenza A virus as determined by the time-of-addition assay. (a) Madin–Darby canine kidney cells were infected with influenza virus A/WSN/33 or A/TW/90206/2009 (multiplicity of infection: 0.01); subsequently, rosmarinic acid (100 μM) was added at the reported time points. (b) Supernatants were collected for a plaque assay; data are means ± standard deviations from three independent experiments. The antiviral activity of rosmarinic acid was calculated with respect to control conditions (infected cells without any treatment) arbitrarily set at 1. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (c) Effect of rosmarinic acid on the hemagglutinin activity of influenza A virus. Guinea pig red blood cells were incubated with influenza virus A/WSN/33 or A/TW/90206/2009 for 1 h in a medium containing different rosmarinic acid concentrations. A virus titer of 4 × HAv was used for the assay, and rosmarinic acid was subjected to two-fold serial dilutions (from 200 μM to 0.4 μM, from left to right). The reported data are from one of three independent experiments.

Results

Rosmarinic acid increases the survival of mice experimentally infected with influenza virus A/WSN/33

To investigate the potential utility of RA for treating IAV infections, its efficacy was investigated in an *in vivo* model. Mice were given either DMSO 5% (100 μL) or RA (100 mg/kg daily) for seven days before an intranasal challenge with influenza virus A/WSN/33. Treatment was subsequently continued for additional ten days after infection. Body weight was measured daily and normalized to the average weight on day 0 (Fig. 1, panel a). RA-treated mice were protected against IAV infection compared with the virus control group (i.e., animals treated with 5% DMSO). The protection was observed both in terms of body weight changes and survival (both p values < 0.05; Fig. 1, panels b and c). Mice treated with RA had a trend towards lower levels of TNF-α in lung homogenates compared with untreated animals ($P = 0.06$; Fig. 1, panel d). Collectively, these results indicate that RA increases the survival of mice experimentally infected with influenza virus A/WSN/33.

Rosmarinic acid inhibits different influenza A virus strains

Using *in vitro* anti-cytopathic effect assays, we examined the CC_{50} and EC_{50} of RA against different IAV strains. As shown in Table 1, RA showed an inhibitory effect against IAV (EC_{50} range: 7.60–70.57 μM). This was especially evident for the five H1N1pdm09 strains. Therefore, RA was more effective against IAV compared with IBV. In addition, RA exerted inhibitory effects against oseltamivir-resistant viruses. To further confirm the antiviral effects of RA, we performed plaque reduction assays against the A/WSN/33 strain and the A/TW/90206/2009 pandemic strain (Fig. 2). The results revealed that RA effectively inhibited both strains in a dose-dependent manner (Fig. 2, panel a). Furthermore, RA was found to decrease viral plaque size — which was three-fold larger in untreated cells (Fig. 2, panel b).

Rosmarinic acid inhibits the early phase of influenza virus A infection

Time-of-addition assays were performed (Fig. 3, panels a and b) in an attempt to clarify the mechanisms by which RA inhibits IAV infections. MDCK cells were treated with RA at various time points with respect to virus adsorption (from −1 to 0 h) — a procedure followed by removal of unbound viruses (Fig. 3, panel a). On analyzing a single infectious cycle, RA was found to predominantly inhibit the influenza virus A/WSN/33 strain and the A/TW/90206/2009 pandemic strain within two time frames (0–12 and −3 to +12 hpi). A marginal inhibition was observed at −3 to −1 hpi, whereas no inhibitory activity was evident for other time frames (−1 to 0, 6–12, and 9–12 hpi; Fig. 3, panel b). We further investigated the ability of RA to inhibit IAV attachment using the HAI assay. As shown in Fig. 3 (panel c), the HAv values of two IAV strains were determined (A/WSN/33 and A/TW/90206/2009). RA up to a non-hemolytic concentration of 50 μM was unable to inhibit RBCs hemagglutination. Collectively, these results suggest that RA inhibits the early phase of IAV infection without affecting virus attachment to host cells.

Rosmarinic acid suppresses viral RNA replication but not RdRp complex activity

We next examined whether RA can interfere with influenza virus RNA expression. The results of a time-course experiment revealed that RA significantly suppressed viral RNA expression as reflected by M1 and NA gene expression levels (Fig. 4, panels a and b). To shed further light on the mechanisms of RA-mediated inhibition of influenza virus RNA expression, we examined its effects on the activity of the RdRp complex using a minigenome assay. The viral PA, PB1, PB2, and NP genes were transfected into HEK293 cells. Compared with the positive control 3P0128, RA was unable to inhibit RdRp complex activity at a concentration of 280 μM.[20] These findings suggest that the RA-elicited inhibition of viral RNA expression is unrelated to a defective RdRp complex activity (Fig. 4, panel c).

The activity of rosmarinic acid against influenza A virus is associated with downregulation of the Akt signaling pathway

Influenza virus infectivity is correlated with altered kinase-regulated signaling pathways.[24] Therefore, we used an antibody-based phosphokinase assay to examine how RA affected kinase activities during a multicycle of infection (Fig. 5). Mock- or influenza virus A/WSN/33-infected A549 cells were treated with either RA or DMSO (negative control). After 24 h, cell lysates were collected and subjected to a phosphoprotein array. Of the 43 proteins examined, the phosphorylation levels of WNK1 (T60), PRAS40 (T246), Src (Y419), Akt (S473), and GSK3α/β (S21/S9) were decreased in RA-treated infected cells compared with those treated with DMSO (Fig. 5, panel a). Using STRING networks, we found that these kinases shared an

Table 1 Inhibition spectra of rosmarinic acid against different viruses — including various influenza A virus strains.

Cell line or virus strain	CC_{50} (μM)[a]	EC_{50} (μM)[b]	Selectivity index[c]
Madin–Darby canine kidney cells	582.02 ± 11.20		
A549	216.82 ± 14.61		
A/WSN/33 (H1N1)		70.57 ± 11.97	8.25
A/CA/07/2009 (H1N1pdm09)		7.60 ± 1.80	76.58
B/TW/99/07		89.61 ± 1.51	6.50
B/TW/70325/05		>500	—
A/Taiwan/6663/2009 (H1N1pdm09)[d]		16.82 ± 1.87	34.60
A/Taiwan/7717/2009 (H1N1pdm09)[d]		26.08 ± 8.70	22.31
A/TW/90206/2009 (H1N1pdm09)		12.22 ± 0.03	47.63
A/TW/90167/2009 (H1N1pdm09)		15.51 ± 3.56	37.53
Adenovirus		>200	—
Human herpes simplex virus type 1		>200	—

[a] CC_{50} was determined with an MTT assay.[21]
[b] EC_{50} was assessed using an anti-cytopathic effect assay.
[c] CC_{50}-to-IC_{50} ratio.
[d] Oseltamivir-resistant strain.[19]
Madin–Darby canine kidney cells were used for influenza virus infections, whereas adenovirus and human herpes simplex virus type 1 infections were performed on A549 cells. Values are means ± standard deviations from two independent experiments.

Akt-centered biological connection (Fig. 5, panel b).[25] Src is implicated in the activation of Akt[26,27] — which in turn modulates the kinase activities of WNK1, PRAS40, and GSK3α/β. Therefore, we used western blotting to investigate the phosphorylation levels of Akt, PRAS40, and GSK3β in a single infectious cycle. Compared with cells treated with DMSO (negative control), cells exposed to RA showed decreased Akt and PRAS40 phosphorylation; this effect was observed in both infected and non-infected cells (Fig. 5, panels c and d). Given the key role played by Akt signaling in virus entry and replication,[28] these results indicate that RA inhibits IAV infections by interfering with this pathway. In addition, RA treatment reduced the amount of both non-phosphorylated and phosphorylated GSK3β (Fig. 5, panel e).

Rosmarinic acid may inhibit influenza A virus entry into host cells by decreasing GSK3β and phospho-AKT expression

GSK3β — an evolutionarily conserved serine/threonine protein kinase with multiple physiological activities — is characterized by constitutive cell expression.[29] Downregulation of GSK3β expression through RNA silencing has been previously found to abrogate IAV entry into host cells.[30] We therefore examined the potential role of GSK3β downregulation in RA-induced inhibition of IAV infections. To this aim, we performed a viral entry assay to assess whether RA is capable of inhibiting IAV entry. A549 cells were pretreated with RA for 16 h and virus adsorption was conducted on ice for 1 h, followed by removal of unbound viruses. Total RNA was extracted following 1 h of viral internalization at 37 °C. The results revealed that cells pretreated with RA had decreased viral RNA levels (Fig. 6, panel a). Because NA plays a key role in IAV entry into host cells,[7,31,32] we subsequently examined the potential effect of RA on viral NA activity. As expected, treatment with host cells with zanamivir significantly inhibited NA activity in IAV-infected cells; however, this was not observed when IAV-infected cells were exposed to different concentrations of RA (Fig. 6, panel b). Collectively, these results indicate that RA inhibits IAV entry into host cells by targeting specific intracellular signaling

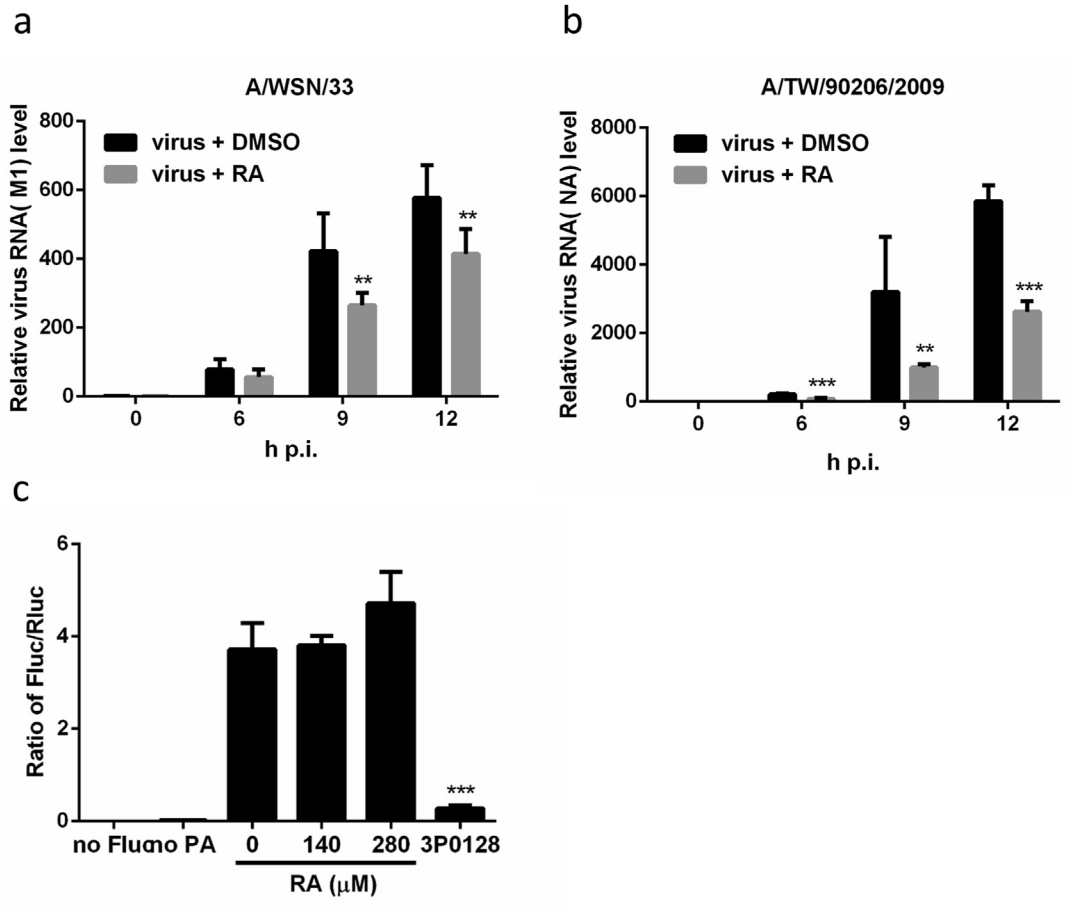

Figure 4. Effects of rosmarinic acid on viral RNA synthesis and RdRp complex activity. HEK293 cells infected with influenza virus A/WSN/33 (a) or A/TW/90206/2009 (b) at a multiplicity of infection of 0.01 were incubated without or with rosmarinic acid (100 μM). Total mRNA was extracted at 0, 6, 9, and 12 h post infection for viral RNA quantification (c). Cells were transfected for 8 h with plasmids encoding PA, PB1, PB2, NP, firefly luciferase (Fluc), and Renilla luciferase (Rluc) prior to the addition of the reported inhibitors. Cell lysates were harvested after 24 h and luciferase activity was quantified. Data are means ± standard deviations from three independent experiments. Each group included four repeats; Fluc activity was normalized to Rluc values. **$P < 0.01$ and ***$P < 0.001$.

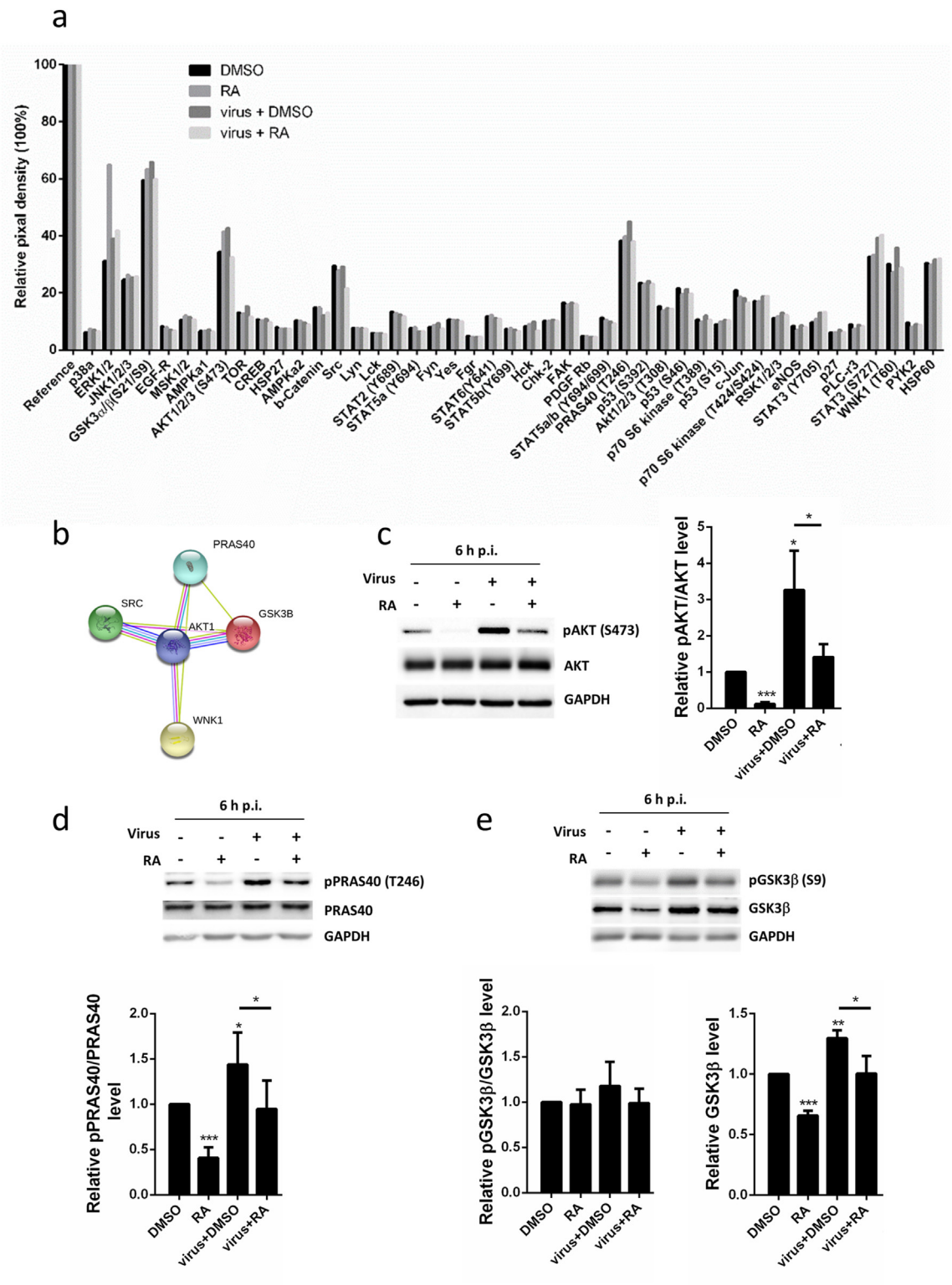

Figure 5. Effects of rosmarinic acid on the phosphoprotein profile of cells infected with influenza A virus. A549 cells infected with influenza virus A/WSN/33 (a) A/WSN/33 at a multiplicity of infection of 0.01 were incubated either without or with rosmarinic acid (100 μM) for 24 h and subsequently analyzed with a human phospho-antibody array. Expression levels of phosphoproteins are presented as relative pixel densities. (b) STRING analysis identified Akt as the hub protein within a network consisting of Akt, WNK1, PRAS40, Src, and GSK3β. (c—e) Effect of rosmarinic acid on Akt, PRAS40, and GSK3β phosphorylation. A549 cells were infected with influenza virus A/WSN/33 (multiplicity of infection: 0.01) in presence of rosmarinic acid or DMSO. Uninfected cells served as controls. Cell lysates were harvested at 6 h post infection for western blot analysis. Data are means ± standard deviations from three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

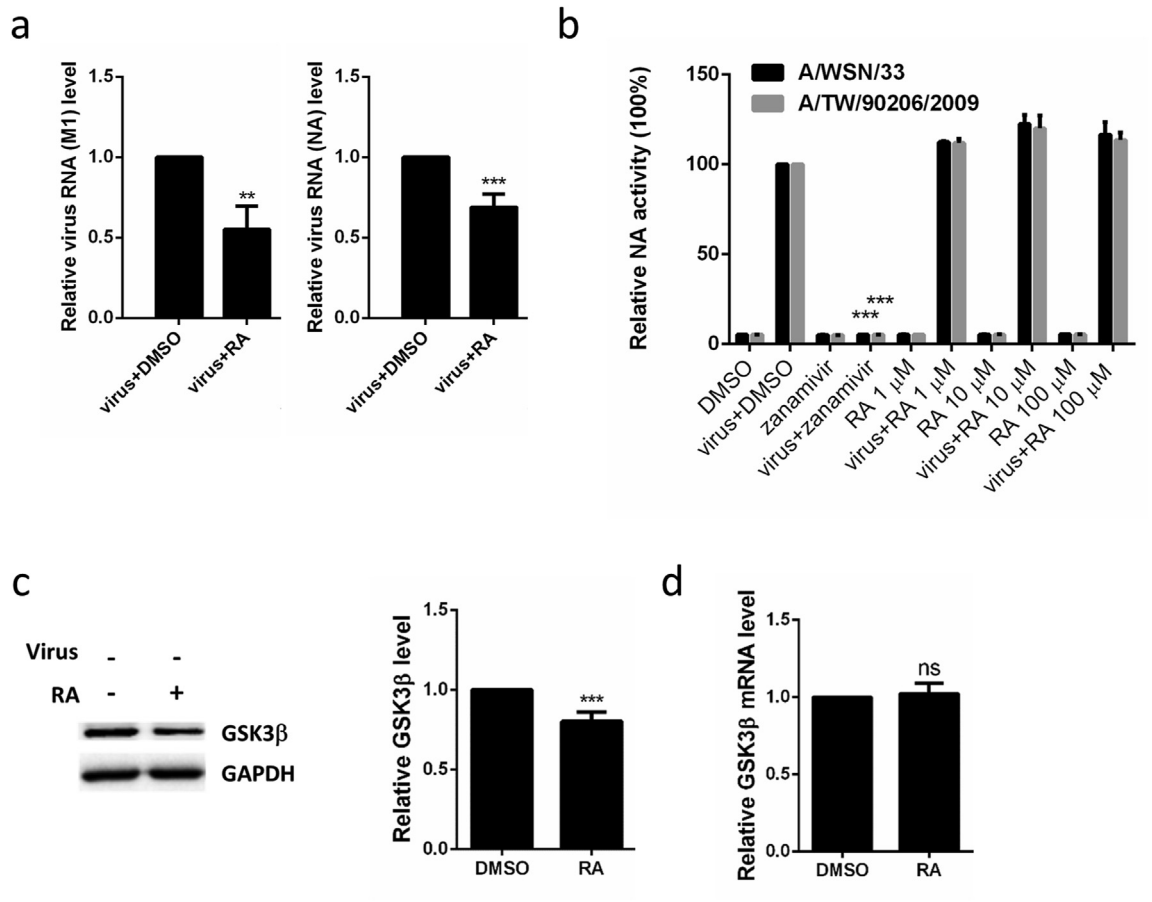

Figure 6. Rosmarinic acid may inhibit the entry of influenza A virus into host cells by downregulating GSK3β expression. (a) Following exposure to rosmarinic acid (100 μM) for 16 h, A549 cells were infected on ice with influenza virus A/WSN/33 (multiplicity of infection: 0.01) for 1 h. After one hour of viral internalization at 37 °C and removal of unbound viruses, total RNA was extracted. (b) Influenza viruses A/WSN/33 or A/TW/206/2009 were incubated with DMSO (1, 2), 0.5 μM of zanamivir (3, 4), or 1 (5, 6), 10 (7, 8), or 100 μM (9, 10) of rosmarinic acid for 30 min. Following the addition of MU-NANA (60 μM), the reaction was incubated at 37 °C for one additional hour. The reaction was terminated through the addition of a stop solution before fluorescence quantification. (c and d) A549 cells were treated with rosmarinic acid (100 μM) for 2 h. GSK3β expression levels were quantified in cell lysates using western blot analysis (c) or from total mRNA using qRT-PCR (d). Data are means ± standard deviations from three independent experiments. **$P < 0.01$ and ***$P < 0.001$. Abbreviation: ns, not significant.

pathways into host cells. Notably, a 2-h treatment with RA was sufficient to decrease the post-transcriptional expression of GSK3β (Fig. 6, panels c and d).

Discussion

Despite decades of research for what remains a significant public health concern, influenza outbreaks still pose major therapeutic challenges. The identification of novel treatment options is therefore required for improving our capacity to more optimally tailor interventions during future epidemics. In the current study, we found that RA increased survival and prevented body weight loss in IAV-infected mice (Fig. 1). Moreover, *in vitro* anti-cytopathic effect assays revealed that RA successfully inhibited different IAV isolates, including oseltamivir-resistant strains (Table 1). On analyzing time-of-addition assays, we also found that RA interferes with the early phases of IAV infection. From a mechanistic point of view, RA downregulated the GSK3β and Akt signaling pathways — which are known to facilitate IAV entry and replication into host cells (Figs. 5 and 6).[33–35]

Using genome-wide RNA interference screening, König et al.[30] showed that several host factors play a crucial role in IAV infection. Of the 295 molecules involved in viral growth, 23 (including GSK3β) were identified as promoters of an efficient virus entry.[30] However, the exact mechanistic role of GSK3β during this process has not been fully elucidated. A previous study reported that dynamin I — a GTPase involved in clathrin-mediated endocytosis (CME) — can act as a direct substrate for GSK3β.[36] GSK3β-mediated phosphorylation of the dynamin I Ser774 residue profoundly affects CME rates, and inhibition of GSK3β activity accelerates the formation and maturation of clathrin-coated pits.[37,38] Given the role played by receptor-mediated endocytosis during IAV entry, more studies are necessary to investigate whether GSK3β inhibition-induced dysregulation of endocytosis may decrease the amount of surface

receptors — ultimately limiting virus entry into the host cell. However, our findings do not rule out the possibility that GSK3β can affect IAV infectivity through mechanisms unrelated to virus entry.

Protein abundance depends on transcription, translation, mRNA decay, and protein degradation. Our data revealed that treatment with RA for 2 h decreased the expression of GSK3β, a long-lived protein, at the protein but not the mRNA level (Fig. 6, panels c and d). These data indicate that RA may inhibit GSK3β expression at the post-translational level. Previous studies have shown that GSK3β may act as a substrate for calpain[39] and matrix metalloproteinase-2.[40] However, no cleaved GSK3β fragments were observed in RA-treated cells. Collectively, these data indicate that RA may downregulate GSK3β via post-translational modifications, including acetylation,[41] citrullination,[42] and mono-ADP-ribosylation.[43,44]

GSK3β activity plays a crucial role during coxsackievirus, hepatitis C virus, and coronavirus infections.[45–47] For example, GSK3β inhibition decreases the release of coxsackievirus viral progeny without affecting viral protein expression.[45] Additionally, GSK3β inhibitors reduce hepatitis C virus replication by downregulating pro-viral hepatic miR-122 expression.[46] Finally, GSK3β inhibitors can inhibit viral nucleocapsid protein phosphorylation and decrease coronavirus replication[47]; thus, they have sparked interest during the recent coronavirus disease-2019 pandemic.[48,49]

The PI3K-AKT signaling pathway can favor influenza virus infection[50] and Akt phosphorylation has emerged as a promising therapeutic target against IAV infections.[35,51,52] PI3K-AKT inhibition interferes with IAV entry and inhibits both viral RNA synthesis and nuclear export of viral RNP. In the current study, we found that RA treatment decreased endogenous and virus-induced phospho-AKT, as well as the synthesis of viral RNA. However, RA did not affect the nuclear export of viral RNP (data not shown). Collectively, these results suggest that RNP export is a complex phenomenon that cannot effectively be inhibited by RA alone.

While RA holds great promise to inhibit viruses that activate the PI3K/Akt pathway (including HSV-1), it can also facilitate viral infections by inducing certain cellular responses that favor replication in host cells. For example, RA can suppress the unfolded protein response — an event which may in turn promote HSV-1 replication.[53–55] Using time-of-addition, attachment, penetration, and plaque reduction assays, Astani et al. demonstrated that RA displays some activity against HSV-1 attachment to host cells — without affecting viral penetration and replication. These assays showed a reduced HSV-1 titer when viruses were pretreated with RA, suggesting that this molecule may target virus particles *in vitro*.[56,57] Musarrat et al.[58] also found that the envelope glycoprotein B of HSV-1 induces AKT activation to promote virus entry into host cells. These results can explain why RA did not show an anti-herpetic activity in our study. As for the different inhibitory effects of RA on IAV and IBV, IAV was found to activate the PI3K/Akt signaling pathway during the entry and replication steps. However, IBV did not produce a sustained PI3K/Akt activation. While a reduced titer of progeny virus was observed in IBV-infected cells that had undergone pretreatment with the PI3K inhibitor wortmannin, this effect did not occur when cells were exposed to IBV and wortmannin simultaneously.[59] In this scenario, the inability of RA to display anti-IBV effects in our anti-cytopathic effect assay is not surprising. Notably, treatment with a PI3K inhibitor promoted H5N1 virus infection.[60] Collectively, these results indicate that the impact of the PI3K/Akt pathway on influenza viral infection may be strain-specific. In summary, our current findings represent a promising step in understanding the potential usefulness of RA for treating and/or preventing IAV infections. An analysis of RA derivates would also have been interesting; however, a screening of 21 related compounds failed to identify better EC_{50} values than that observed for RA (data not shown). Further studies are required to improve the anti-IAV potency of this phytochemical.

Ethical approval and consent to participate

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Chang Gung University (approval number: CGU10-001).

Consent for publication

Not applicable.

Availability of data and materials

All data relevant to the study are included in the article. Additional information (e.g., protocols) is available from the corresponding author upon reasonable request.

Authors' contributions

J.R. Jheng and C.F. Hsieh contributed equally to this work. Study concept and design: J.R. Jheng, J.T. Horng; Acquisition of laboratory data: J.R. Jheng, C.F. Hsieh, Y.H. Chang, J.Y. Ho, W.F. Tang, Z.Y. Chen, C.J. Liu, T.J. Lin, L.Y. Huang; Data analysis and interpretation: J.R Jheng, J.H. Chern; Drafting of the manuscript and/or critical revision for important intellectual content: J.R. Jheng, C.F. Hsieh, J.T. Horng, J.Y. Ho.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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