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Germacrone inhibits early stages of influenza virus infection

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ABSTRACT

Highly pathogenic influenza viruses pose a serious public health threat to humans. Although vaccines are available, antivirals are needed to efficiently control disease progression and virus transmission due to the emergence of drug-resistant viral strains. In this study, germacrone, which is a major component of the essential oils extracted from Rhizoma Curcuma, was found to inhibit influenza virus replication. Germacrone showed antiviral activity against the H1N1 and H3N2 influenza A viruses and the influenza B virus in a dose-dependent manner. The viral protein expression, RNA synthesis and the production of infectious progeny viruses were decreased both in MDCK and A549 cells treated with germacrone. In a time-of-addition study, germacrone was found to exhibit an inhibitory effect on both the attachment/entry step and the early stages of the viral replication cycle. Germacrone also exhibited an effective protection of mice from lethal infection and reduced the virus titres in the lung. Furthermore, the combination of germacrone and oseltamivir exhibited an additive effect on the inhibition of influenza virus infection, both *in vitro* and *in vivo*. Our results suggest that germacrone may have the potential to be developed as a therapeutic agent alone or in combination with other agents for the treatment of influenza virus infection.

1. Introduction

Due to the emergence of new pandemic strains through viral mutation and reassortment, as exemplified by the 2009 H1N1 influenza pandemic (Peiris et al., 2009), the influenza A virus causes acute respiratory infections in humans, with severities ranging from morbidity to mortality. Annual outbreaks in recent years in the United States alone claimed over 36,000 lives and cost billions of dollars (Molinari et al., 2007; Rothberg et al., 2008). There is concern that the 2009 H1N1 virus will continue to cause serious disease in the immediate future (Retrieved from www.cdc.gov/h1n1flu/). Although annual vaccination is the primary strategy for the prevention of infections, influenza antiviral drugs play an important role in the comprehensive approach for the control of illness and transmission.

Two classes of antiviral drugs, namely M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (NAIs; zanamivir and oseltamivir), have been approved for the treatment and prophylaxis of influenza (De Clercq, 2006). The adamantanes can inhibit M2 ion channel activity by blocking the migration of H^+ ions into the interiors of the virus particles within endosomes, which is a process that is needed for uncoating to occur (Pinto et al., 1992). However, their wide use has been limited due to the rapid emergence of drug resistance, the ready transmissibility of drug-resistant viruses and the occurrence of central nervous system side effects (Weinstock and Zuccotti, 2006). Neuraminidase inhibitors, such as zanamivir and oseltamivir, interfere with the release of progeny influenza virions from the surface of infected host cells (Moscona, 2005). In doing so, the neuraminidase inhibitors prevent the virus infection of new host cells and thereby halt the spread of infection in the respiratory tract (De Clercq, 2004; Matrosovich et al., 2004). Influenza viruses with low susceptibility to NAIs have been isolated *in vitro* and *in vivo*. Resistance involves either a mutation in the active site of the NA, which alters its sensitivity to inhibition, or a mutation in hemagglutinin (McKimm-Breschkin, 2000). The increasing appearance of resistant strains of influenza virus highlights our urgent need to identify new antiviral drugs.

Using herbal formulas to prevent and treat colds and flu is one of the best-developed and most successful aspects of traditional Chinese medicine. It is reasonable to speculate that small molecules with anti-influenza properties exist in certain traditional Chinese herbal medicines. In an effort to identify new antiviral therapies that are effective against influenza viruses, we tested the anti-influenza activity of hundreds of compounds that were isolated from traditional Chinese herbal medicines. Among these compounds, germacrone (Fig. 1A) has been found to inhibit

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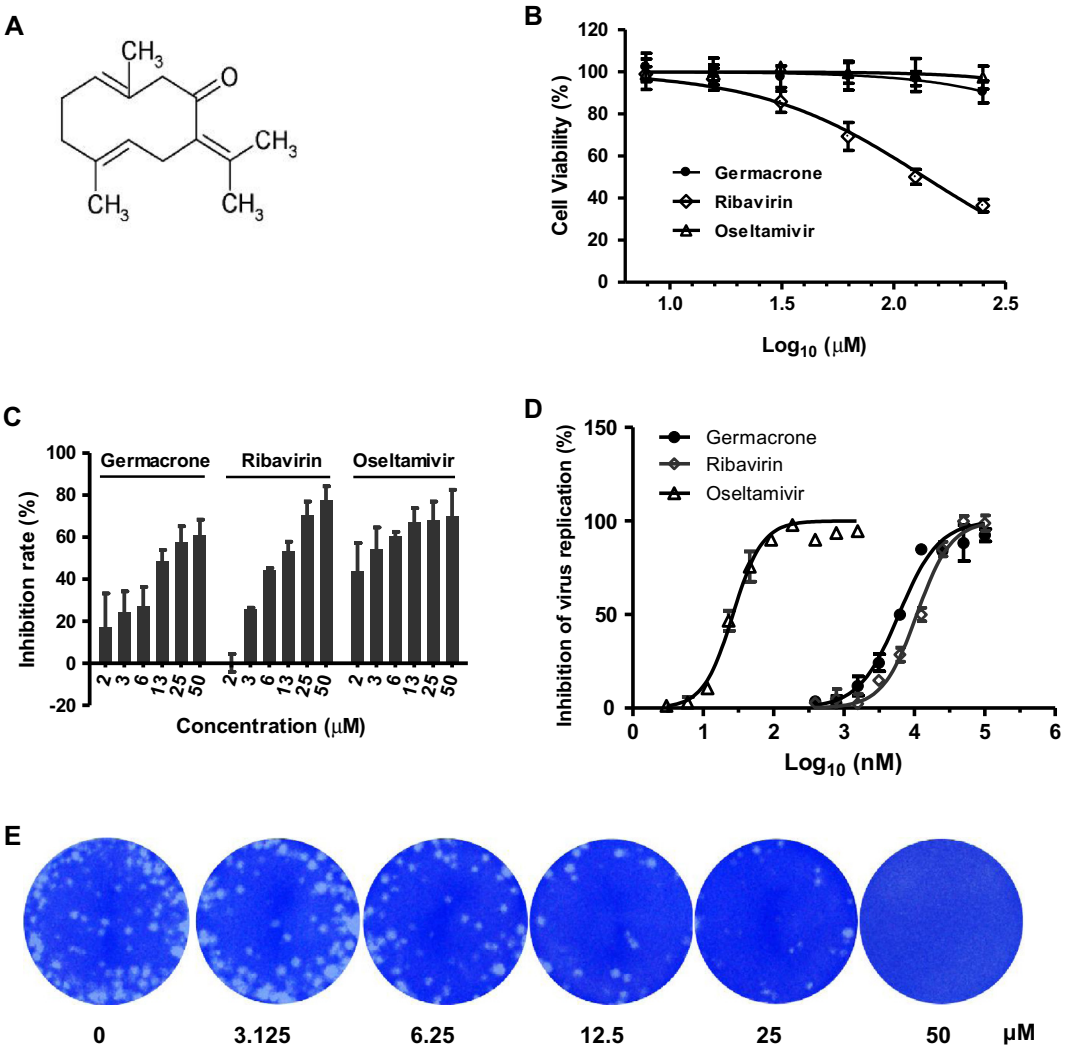

Fig. 1. Germacrone inhibits influenza virus replication. (A) Chemical structure of germacrone. (B) A confluent monolayer of MDCK cells was washed three times with PBS and then incubated in media (with 2% FBS) containing various concentrations of germacrone (closed circle), ribavirin (open diamond), or oseltamivir carboxylate (open triangle). After 48 h of incubation at 37 °C, the cell viability was determined through the MTT assay. The optical densities (OD_{550}) of the compound-treated cells are expressed as a percentage of the viable cells. (C and D) Confluent cells were infected with 100 $TCID_{50}$ of A/PuertoRico/8/34 viruses and after 1 h of incubation at 37 °C, the cells were washed with PBS three times and various concentrations of drug-containing media were added. After 48 h incubation at 37 °C, the cell viability of the drug-treated and non-treated cells, which represents the protection against a cytopathogenic effect, was measured through the MTT method (C). The supernatants in the cells were collected and used in the neuraminidase activity assay (D). In both assays, the readouts from the uninfected cells were taken to be 100% and the readouts from the infected cells treated with the DMSO control were taken to be 0%. The bar indicates the SDM of three different experiments. (E) Germacrone-induced reduction in plaque formation. Details of the seeding of MDCK cells in six-well plates and the infection with influenza virus are described in the plaque reduction assay section of Materials and Methods. At 96 h post infection, the cell monolayers were fixed and stained to show the plaques.

influenza viruses *in vitro*. Further studies have shown that germacrone interferes with the early stages of the viral lifecycle in cells and protects mice from lethal infection. Germacrone is one of the main active ingredients in the essential oils extracted from Rhizoma Curcuma (Ezhu in Chinese), which is widely prescribed in traditional Chinese medicine for anti-tumour therapy. Our findings suggest that the compound germacrone, its derivatives, or Rhizoma Curcuma might be as potential treatments for influenza virus infection.

2. Materials and methods

2.1. Viruses and cells

The A/PuertoRico/8/34(H1N1), A/human/Hubei/1/2009(H1N1), A/human/Hubei/3/2005(H3N2), A/human/WSN/33(H1N1, S31N, amantadine resistant) and influenza B virus B/human/Hubei/1/2007 were propagated in the allantoic cavity of 10-day-old specific-pathogen-free embryonated chicken eggs at 37 °C. The allantoic fluid was harvested, centrifuged for clarification and stored at −70 °C (Rajik et al., 2009). The virus titre was determined through a hemagglutination test (HA) and through the analysis of the 50% tissue culture infective dose ($TCID_{50}$) in Madin-Darby Canine Kidney Cells (MDCK) cells and evaluated using the method developed by Reed and Muench (1938). The MDCK cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) and a human pulmonary epithelial cell line (A549 cells) was cultured in RPMI 1640 supplemented with 10% foetal bovine serum (Invitrogen, CA, USA) and antibiotics at 37 °C in 5% CO_2. After influenza virus infection, the cells were cultured in infection medium (DMEM or 1640 containing 0.3% bovine serum albumin and 2.5 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO, USA)).

2.2. Reagents

Germacrone was provided by Sichuan Weikeqi, Co. (Chengdu, China). Ribavirin and oseltamivir (GS 4071) were obtained from Sigma Chemical Company (Sigma–Aldrich, MO, USA) and Toronto Research Chemicals (Toronto, Canada) respectively. All test compounds were initially dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich) at a concentration of 10 mM.

2.3. Cytotoxicity tested by the MTT assay

Cells were grown in 96-well plates for 16 h. The medium was replaced with fresh medium containing serially diluted compounds and the cells were further incubated for 48 h. The culture medium was removed and replaced with 100 μl 3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide (MTT; Sigma–Aldrich) solution and incubated at 37 °C for 4 h. After removal of the supernatant, a dissolving solution (10% SDS, 5% isobutanol and 0.012 mmol/L HCl in distiled water) was added to all of the wells and mixed thoroughly to dissolve the dark blue crystals. After 4 h incubation at room temperature to ensure that all of the crystals were dissolved, the absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain a sample signal.

2.4. Viral inhibition assays tested by the MTT assay and the neuraminidase activity assay

The antiviral activity of compounds against the influenza virus was determined through the following protocol. Cells were infected with 100 $TCID_{50}$:0.1 ml of the virus in the presence of different concentrations of the studied compounds at 37 °C for 1 h. After virus attachment, the cells were washed three times with D-Hank's solution and incubated with media containing the same concentration of the studied compounds at 37 °C for 48 h. For the neuraminidase activity assay, the cells were cultured in medium containing the studied compounds for 12 h and then in fresh medium without the studied compounds for an additional 36 h. In the primary screening, the inhibition of viral replication was measured by determining the protection against a cytopathogenic effect, which was indicated by the cell viability. For the confirmation of the inhibition of viral replication, a standard fluorimetric assay was used to measure the influenza virus neuraminidase activity of the supernatant (Wetherall et al., 2003). In this assay, the substrate [2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid] is cleaved by neuraminidase to yield a fluorescent product that can be quantified. The fluorescence intensity that resulted from the substrate cleavage by the viral neuraminidase activity was measured at 355 nm (excitation) and 485 nm (emission) using a multilabel plate reader (Wallac Envision, PerkinElmer, MA, USA). The effective concentrations that induced 50% inhibition of the viruses (EC_{50}s) were calculated through a nonlinear regression analysis (sigmoidal dose–response variable slope) using the GraphPad Prism 5.0 software.

2.5. Plaque reduction assay

MDCK cells were seeded in 6-well plates at a density of 6×10^5 cells per well. Twenty-four hours later, the cells were washed with PBS and infected with the supernatants collected from virus-infected cells maintained in the presence of different concentrations of germacrone for 1 h at 37 °C. The virus inoculums were removed by washing with PBS. The cell monolayers were then overlaid with agar overlay medium (DMEM supplemented with 1% low melting point agarose, 0.3% bovine serum albumin and 2.5 μg/ml TPCK-treated trypsin) and incubated at 37 °C for 3–4 days. The cell monolayers were fixed with 4% paraformaldehyde for 1 h. The culture media and agarose overlay were then removed and the cell monolayers were stained with 2% (w/v) crystal violet prepared in 10% ethanol.

2.6. Infectious virus yield reduction assay

Triplicate MDCK cell monolayers in 24-well plastic plates were challenged with 100 $TCID_{50}$:0.25 ml of the virus in the presence of different concentrations of germacrone. After a 1 h adsorption at 37 °C, the monolayers were washed twice with phosphate buffered saline (PBS) and incubated at 37 °C in the same compound-containing medium for 48 h. The yields of the infectious virus at the indicated time points in the supernatant were titrated through the $TCID_{50}$ assay and the virus titre was calculated using the Reed–Muench method.

2.7. Indirect immunofluorescence assay

Cells seeded at a density of 5×10^4 on glass coverslips were inoculated with medium alone, virus suspension, or virus mixed with various concentrations of compound at 4 °C for 1 h, then incubated for 6 h at 37 °C. Cells were washed twice with PBS 6 h after infection and fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. The cells were then incubated in blocking buffer (PBS containing 3% BSA, 0.3% Triton X-100 and 10% FBS) for at least 30 min and then in binding buffer (PBS containing 3% BSA and 0.3% Triton X-100) with monoclonal antibodies against the nucleoprotein (1:50, Santa Cruz, CA, USA) for 1 h. After further washes, cells were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin G (1:200) and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich). The stained samples were then examined with a ZEISS Axio Observer A1 microscope.

2.8. Semi-quantitative RT-PCR analysis

The protocols used for RNA isolation and the semi-quantitative reverse transcription PCR analysis of the intracellular RNA were described by Song et al. (2005), although some modifications were implemented. The cells were grown to approximately 90% confluence, infected with 100 $TCID_{50}$ of influenza A virus and cultured in the presence of the compound of interest. At 6 h post infection (hpi), the cells were scraped off and collected by centrifugation (12,000 *g* for 5 min). The cell pellets were washed twice with PBS. The total cellular and viral RNAs were extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The first-strand cDNA was synthesised from 1 μg of total RNA with an AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) using oligo(dT) primers. The PCR reactions were performed with 50 μl of reaction buffer [5 μl of cDNA template, 50 pmol of the primers, 0.1 mM dNTPs and 0.5 U of rTaq polymerase (Takara)]. The amplification conditions were the following: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 40 s and 72 °C for 2 min, and; 1 cycle of 72 °C for 5 min. The NP RNA was chosen for detection and the primer sequences used for the detection of viral RNA were 5′-TGC TGG ATTA CTC GTT CGG TC and 5′-CCT TTA TGA CAA AGA AGA AAT AAG GCG. Actin was used as an internal control of the cellular RNAs using the following primer sequences: 5′-TCA CCC GAGA TCC ATC ACG AT and 5′-GAA GTA CCC CAT TGA GCA CGGAAAAA.

2.9. Time-of-addition and inhibition time course assay

A time-of-addition experiment was performed as previously described (Furuta et al., 2005) with some modifications. The confluent monolayers of cells were inoculated with 100 $TCID_{50}$ of virus. After attachment at 4 °C for 1 h, the monolayer cells were washed three times with PBS and incubated in DMEM containing 0.3% bovine serum albumin and 2.5 μg/ml of trypsin in 5% CO_2 at 37 °C. For the neuraminidase activity assay, the media (with or without various concentrations of the compounds of interest) were added at −2 h (before virus attachment), −1 h (at the time of attachment at 4 °C), 0 h (after virus attachment at 4 °C), 1, 2, 3, 4, 5 and 6 h and the cells were incubated for 48 h at 37 °C. The supernatants were then collected and used in the neuraminidase activity assay. For the inhibition time course assay using an indirect immunofluorescence assay, the media (with or without various concentrations of compounds) were added during the periods of −2 to −1 h (treatment of cells before attachment), −1 to 0 h (attachment period at 4 °C), 0–2 h, 2–4 h and 4–6 h (replication period at 37 °C). After each incubation period, the monolayers were washed three times with PBS, incubated with fresh medium and cultured at 37 °C. At 6 hpi, the monolayers of the cells were fixed with 4% paraformaldehyde prior to the determination of the viral yield through an indirect immunofluorescence assay.

2.10. In vivo antiviral studies

The experiments were conducted according to the protocol approved by the Animal Care and Use Committee of Wuhan Institute of Virology of the Chinese Academy of Sciences (WIVA08201202). Specific-pathogen-free BALB/c mice weighing approximately 18–20 g were purchased from Changsha Laboratory Animal Centre in Hunan Province, China. The mice were maintained on standard rodent chow, given water *ad libitum* through water bottles and quarantined 48 h prior to the onset of the studies. The mice were anesthetised through the intraperitoneal injection of sodium pentobarbital (75 mg/kg) and then intranasally infected with 5 LD_{50} of influenza virus (A/PuertoRico/8/34). Germacrone (50 mg/kg or 100 mg/kg) was administered intravenously once daily for 5 days, starting 24 h before virus exposure. Oseltamivir (50 mg/kg, positive control) and the solvent (a negative control) were administered once daily through the oral and intravenous routes, respectively, for 6 days. The survival of the infected animals (16 in each group) was monitored daily for 18 days.

On days 2 and 4 post infection, three mice were sacrificed from each group. The trachea and lungs were removed and washed three times through the injection of 2 ml of PBS containing 0.1% BSA. After the cellular debris was removed by centrifugation at 3000*g* for 10 min, the supernatants (the bronchoalveolar lavage fluid, BALF) were subjected to virus titration. The virus titres of the BALF were measured through the $TCID_{50}$ assay and calculated through the Reed–Muench method.

2.11. Drug combination studies

The combination of germacrone and oseltamivir was evaluated in cells through MTT assay and in mice through a survival assay, as described above. MDCK cells were infected with 100 $TCID_{50}$:0.1 ml of the virus in the presence of different concentrations of germacrone and oseltamivir (either alone or in combination) at 37 °C for 1 h. After virus attachment, the cells were washed three times with D-Hank's solution and incubated with fresh media containing the same concentration of drugs at 37 °C for 48 h. The inhibition of viral replication was measured through the MTT assay. The extent of the effect of the combination treatment was analysed through the isobole method (Berenbaum, 1989). The fractional inhibitory concentrations (FICs) were calculated by dividing the EC_{50} of drug A with a fixed overlay of drug B by the EC_{50} of drug A alone (the *x*-coordinate). They-coordinate is the fixed concentration of drug B divided by the EC_{50} of drug B alone. The FIC index is the sum of a pair of FICs. For the *in vivo* study, the mice administered germacrone (100 mg/kg) and oseltamivir (1 mg/kg) once daily for 6 days either alone or in combination. The survival of the infected mice (13 in each group) was monitored daily for 18 days. On day 4 post-infection, three mice were sacrificed from each group and the collected BALF was applied to virus titration through the $TCID_{50}$ assay.

2.12. Statistics

The software GraphPad Prism (Version 5.0) was used for all statistical analysis and graphical illustrations. Data were presented as means ± standards deviation for at least three independent experiments. The statistical significance was analysed by using Student *t*-test. The significant difference was considered as $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Germacrone can inhibit influenza virus replication

In a screen of antivirals from natural products used in Chinese herbal medicines based on the MDCK cell infection model, germacrone (Fig. 1A) was found to inhibit the influenza virus in a dose-dependent manner. As shown in Fig. 1C and D, treatment of MDCK cells with serially diluted germacrone at concentrations not exceeding the maximum non-toxic dose (MNTD; Fig. 1B) at the same time as the inoculation of the influenza A/PR8/34 (H1N1) virus resulted in a dose-dependent increase in the inhibition rate, which was represented both by the cell viability (cytopathic effect inhibition, Fig. 1C) as determined by the MTT assay and by the neuraminidase activity (Fig. 1D) measured through the neuraminidase assay. Similar to the reference compounds oseltamivir and ribavirin, germacrone exhibited a dose-dependent inhibition of the influenza virus. Based on the dose–response curves shown in Fig. 1D, the EC_{50} for germacrone is approximately 6.03 μM. The neuraminidase assay was also performed in A549 cells (Fig. 1S) and the incubation with germacrone decreased the virus-induced neuraminidase release into the supernatants in a dose-dependent manner, whereas the incubation with ribavirin exhibited no antiviral effect. To evaluate the antiviral activity of germacrone on the production of infectious influenza viruses, the plaque reduction assay was performed. Our results showed that germacrone can reduce the plaque numbers in a dose-dependent manner compared with the DMSO control (Fig. 1E). All of these results demonstrate that germacrone has antiviral activity against the influenza virus.

3.2. Germacrone can reduce the influenza virus RNA transcription, protein expression and progeny virus production

To determine whether germacrone inhibits the transcription of viral RNA in infected cells, semi-quantitative RT-PCR was performed to measure the production of specific viral mRNA. Cells were infected with the A/PR/8/34 virus and incubated for 6 h in the presence or absence of the compounds of interest. The total RNA was isolated from the infected cells and analysed by RT-PCR using primers specific for the viral NP mRNA. As shown in Fig. 2A, similarly to ribavirin, which is a known inhibitor of influenza virus RNA synthesis, the addition of germacrone to MDCK cells decreased viral RNA synthesis. In contrast, only germacrone significantly decreased the viral RNA synthesis in A549 cells. The transcription of cellular β-actin, which was measured as an

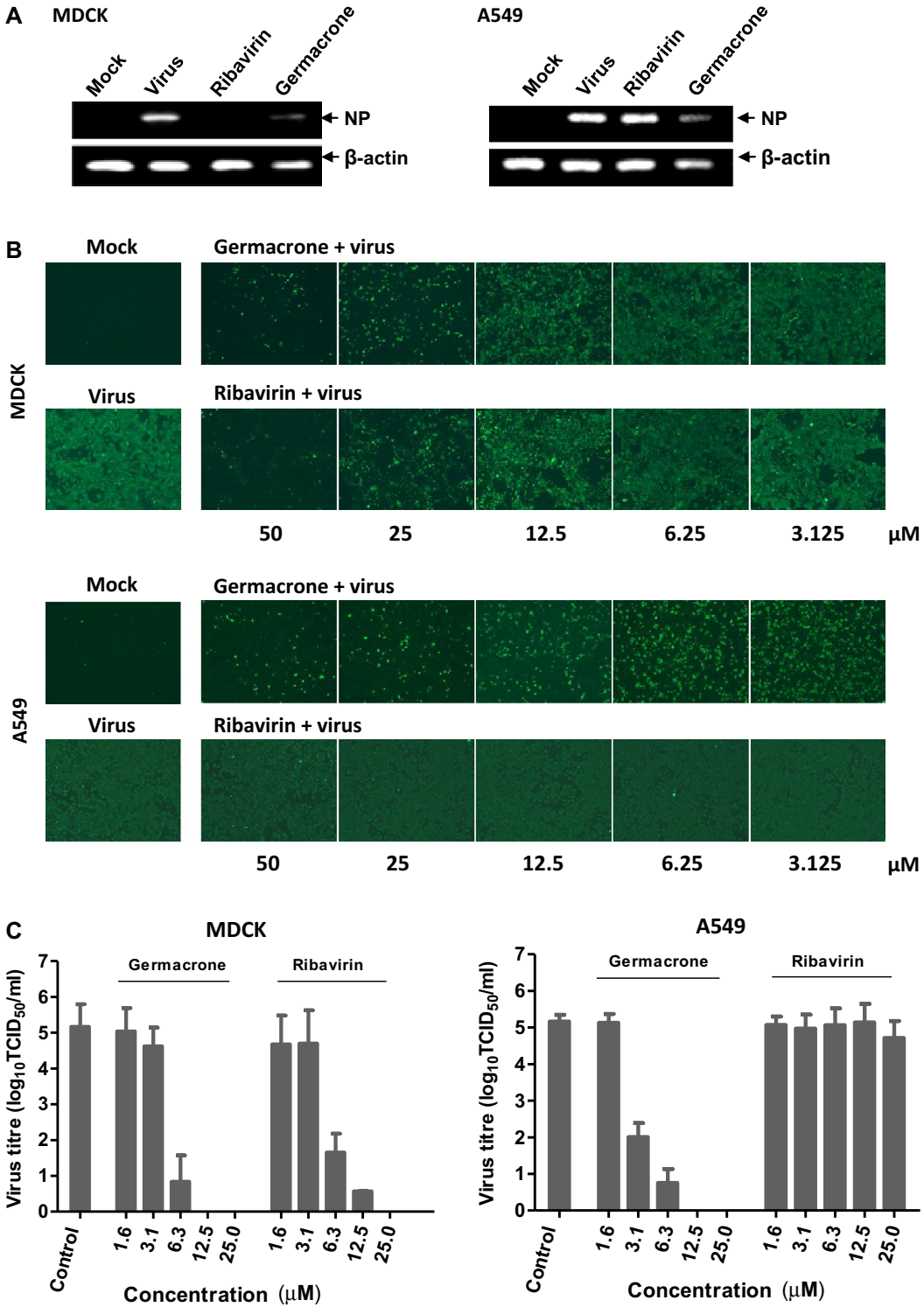

Fig. 2. Germacrone impairs viral RNA synthesis, protein expression and the production of infectious progeny viruses in MDCK cells. (A) Confluent cells were incubated with the A/PuertoRico/8/34 viruses for 1 h and then treated with 50 μM (MDCK cells) or 25 μM (A549 cells) germacrone, ribavirin, or oseltamivir. After incubation at 37 °C for 6 h, the cells were lysed and the total RNA was extracted. RT-PCR was performed with specific primers for viral RNA (NP) and cellular RNA (β-actin) as an internal control and analysed by agarose gel electrophoresis. (B) Confluent cells were infected with the A/PuertoRico/8/34 viruses and then treated with various concentrations of germacrone or ribavirin. At 6 hpi, the cells were fixed with paraformaldehyde and the influenza virus NP expression was detected by IFA using a monoclonal NP antibody. (C) Triplicate monolayers in 24-well plastic plates were challenged with 100 $TCID_{50}$:0.1 ml of the virus in the presence of different concentrations of germacrone or ribavirin. After 1 h adsorption at room temperature, the monolayers were washed twice with PBS and incubated at 37 °C in fresh media containing the same drug cocktail for 48 h. The production of infectious virus in the supernatants was titrated through the $TCID_{50}$ assay. The error bar indicates the SD of three different experiments.

internal control, was not affected by any of the treatments; therefore, the observed inhibitory effect on viral RNA synthesis was not due to a general cytotoxic effect of germacrone on the cells.

To confirm that germacrone impairs viral protein synthesis, the expression of the viral nuclear protein was analysed through an indirect immunofluorescence assay. As shown in Fig. 2B,

germacrone (top panel) exhibited the same level of dose-dependent inhibition of the NP protein synthesis as ribavirin (lower panel) in MDCK cells. In contrast, only germacrone (top panel) showed a significant dose-dependent inhibition of NP protein synthesis in A549 cells, whereas ribavirin (lower panel) exhibited a significantly lower inhibition of NP protein synthesis.

To measure the efficacy of germacrone in inhibiting the production of infectious progeny viruses, the infectivity of the viruses in the supernatants of cells treated with different concentrations of germacrone at the indicated time points was determined through the $TCID_{50}$ assay. As shown in Fig. 2C, in MDCK cells, germacrone, similarly to ribavirin, exhibited a dose-dependent inhibition of the infectivity represented by the virus titre of infectious progeny viruses. In contrast, in A549 cells, only germacrone showed a significant inhibition of the infectivity and ribavirin showed no inhibition of the infectivity.

Taken together, these data demonstrate that germacrone can impair viral RNA replication, protein synthesis and the production of infectious progeny viruses and can inhibit influenza virus replication in both MDCK and A549 cells.

3.3. Germacrone inhibits the influenza virus in the early stages of its lifecycle

To identify the step(s) of the influenza lifecycle inhibited by germacrone, we performed time-of-addition and time course studies of the inhibitory effects of germacrone. It has been reported that at least 6 h are required to detect a progeny virus after inoculation of the influenza A/PR/8/34 virus (Kamps et al., 2006). In addition, when the cells are infected at 4 °C, only virus attachment occurs. Virus penetration occurs only when the cells are cultured at 37 °C after virus attachment. In the neuraminidase activity assay, the compound was applied starting 1 h before the attachment, just when attachment at 4 °C starts and every 1 h during the first 6 h of infection at 37 °C to cover the whole lifecycle of this virus strain from absorption to progeny production. The supernatants were collected for the neuraminidase activity assay. As shown in Fig. 3A, apparent germacrone-induced inhibition of virus amplification was observed when two infected cells were treated −2, −1, 0, 1 and 2 h post virus absorption, although the inhibition in the MDCK cells is slightly less than that observed in the A549 cells at 1 and 2 hpi. This time frame covers the early steps of the influenza virus lifecycle, such as attachment, internalisation and fusion. The result suggests that germacrone may inhibit both the attachment and the penetration of the influenza virus.

To further confirm that germacrone inhibits the early steps of the influenza virus lifecycle, we chose five time intervals (−2 to 1, −1 to 0, 0–2, 2–4 and 4–6 h) for the inhibition time course by IFA to detect the expression of the NP protein. As shown in Fig. 3B, a significant inhibition of virus replication, as represented by the expression of NP, was observed when germacrone was added to the cells within −2 to 4 h of viral infection in both MDCK and A549 cells. Germacrone did not exert its antiviral activity when it was added more than 4 h after infection, which indicates that germacrone is ineffective during the late stages, i.e., assembly and release, of the viral lifecycle. Interestingly, our results showed that ribavirin inhibits influenza virus replication only within the time frame of 0–4 h in MDCK cells and exhibits no apparent inhibition of viral replication in A549 cells throughout the lifecycle of the virus. Taken together, these results suggest that germacrone blocks multiple steps in the early stages of the influenza virus lifecycle. It is important to note that germacrone can inhibit viral replication by targeting the cells because pretreatment of the host cells with germacrone markedly impairs the infection of the influenza virus (Fig. 3B).

3.4. Inhibitory effect of germacrone on different strains of influenza viruses

To test the inhibitory effect of germacrone on other influenza virus strains, various concentrations of germacrone were tested for their antiviral activities against the influenza A/human/Hubei/1/2009(H1N1), A/human/Hubei/3/2005(H3N2), A/human/WSN/33(H1N1, S31N, amantadine resistant) and influenza B viruse B/human/Hubei/1/2007. MDCK and A549 cells were infected with 100 $TCID_{50}$:0.1 ml of the virus and incubated for 48 h in the presence of the compounds (or none as a control). The viral replication levels were then determined by measuring the neuraminidase activity. As shown in Fig. 4, germacrone exhibited a dose-dependent inhibition of all four viral strains, including an adamantane-resistant virus A/WSN/33/S31N(H1N1), both in MDCK and A549 cells. In contrast, ribavirin exhibited an antiviral effect in MDCK cells but not in A549 cells. The EC_{50} values for the inhibition of virus replication were calculated and are presented in Table 1.

3.5. Inhibitory effect of germacrone on influenza virus in vivo

To evaluate the efficacy of germacrone against influenza virus *in vivo*, BALB/c mice intranasally infected with 5 LD_{50} of the virus were intravenously administered germacrone once daily for 6 days, starting one day before infection. Infected mice treated with oseltamivir were used as a positive control. The mouse survival was monitored daily for 18 days. As shown in Fig. 5A, the number of surviving animals increased with germacrone administration in a dose-dependent manner. We observed 50% survival when 100 mg/kg of germacrone was administered and 10% survival when 50 mg/kg of germacrone was administered. In addition, there was a significant delay in the mortality time in mice treated with germacrone compared with the untreated mice.

On days 2 and 4 post infection, the lungs of the mice were isolated and the BALF was collected. The virus titres in the BALF were then determined on MDCK cells through $TCID_{50}$ analysis. As shown in Fig. 5B, the virus titres were significantly lower at 2 dpi in the groups treated with 50 and 100 mg/kg of germacrone. In contrast, at 4 dpi, only the mice treated with 100 mg/kg of germacrone exhibited a significant decrease in virus titres and the mice treated with 50 mg/kg of germacrone showed a slightly decrease. At 100 mg/kg, germacrone alone did not cause mouse death or body weight loss, indicating that the antiviral effect on mice was not due to the drug toxicity. The mice in the oseltamivir group exhibited a significant decrease in virus titres at both 2 and 4 dpi. Taken together, our data suggest that germacrone can inhibit the influenza virus *in vivo*.

3.6. Germacrone and oseltamivir exhibit an additive effect on influenza virus infection

Because germacrone strongly inhibits influenza virus replication in cells and shows moderate antiviral effect *in vivo*, it could be a potentially used in combination therapy by inhibiting influenza virus infection and delaying the emergence of drug-resistant virus. Germacrone was tested with oseltamivir *in vitro* and *in vivo* for combination therapy.

The combination study in cells was conducted using a checkerboard setup in which dose–response curves were generated for oseltamivir either alone or in combination with germacrone (Fig. 6A) and the EC_{50} values for each drug alone or in the presence of a fixed concentration of the second drug were calculated. Isobologram analysis (Berenbaum, 1989) and the fractional inhibition concentration index (FIC index; Odds, 2003) were used to determine whether the combination of oseltamivir and germacrone

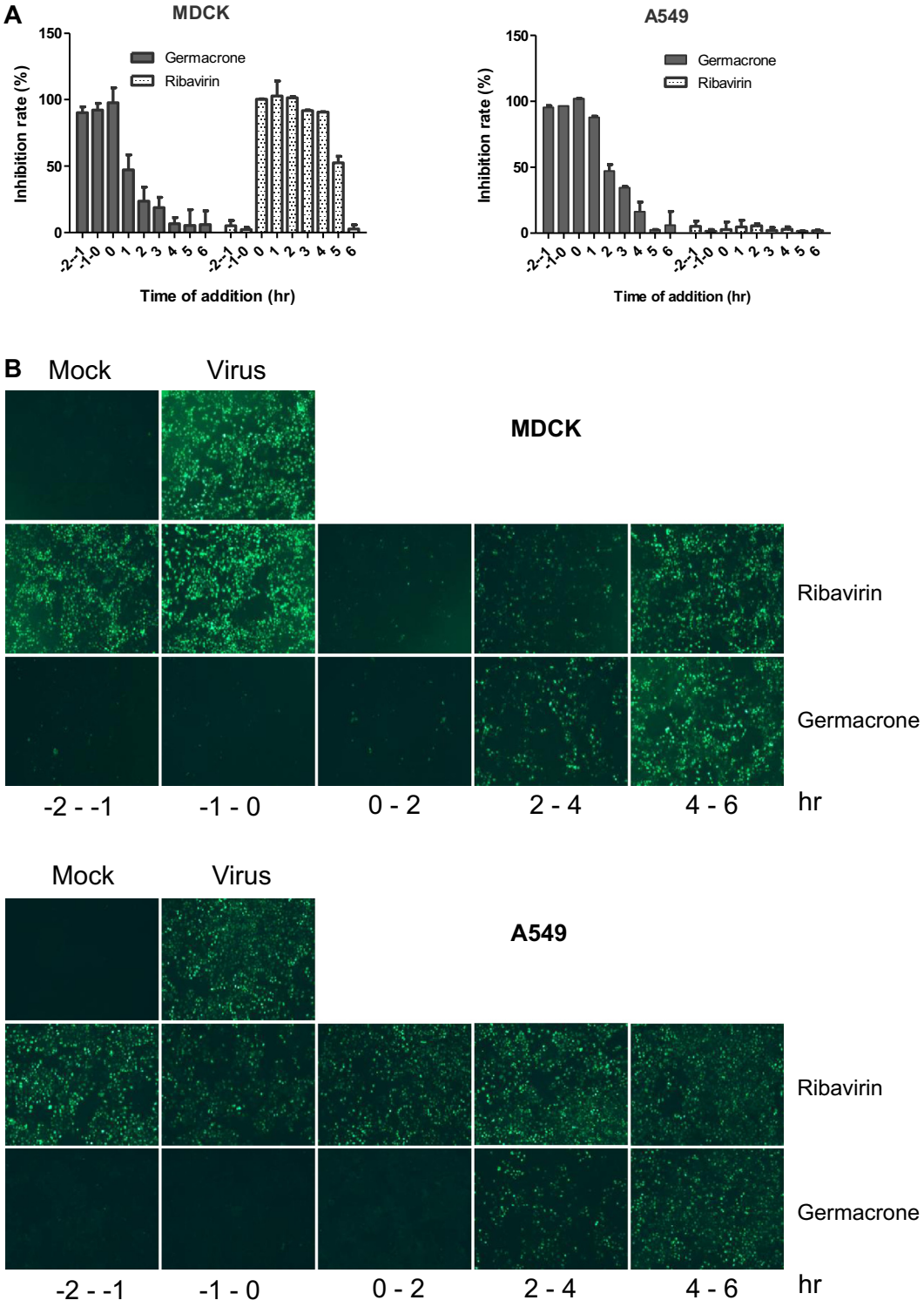

Fig. 3. Inhibitory effect of germacrone on the specific steps of the virus lifecycle. In a time-of-addition assay, cells were inoculated with 100 $TCID_{50}$:0.1 ml of the influenza A/PuertoRico/8/34 virus. (A) 25 μM of germacrone or ribavirin was added at the indicated times and maintained until harvest. The viral infection was performed between −1 and 0 h at 4 °C. After 48 h of incubation at 37 °C, the neuraminidase activity in the supernatants was measured. The bar indicates the SD of three different experiments. (B) 25 μM of germacrone or ribavirin was added at the indicated times and removed after 2 h. After each incubation period, the cells were incubated with fresh media until 6 h post infection. The cells were then fixed and the viral replication was determined through IFA using a monoclonal antibody against NP.

exerts a synergistic, additive, or antagonistic effect on the inhibition of influenza virus infection. As shown in Fig. 6B, the *x*-axis and *y*-axis represent the FICs of oseltamivir and germacrone, respectively. The calculated effects of the combination therapy, which is shown by the closed circles located near the additive line or by the calculated FIC indexes, which are all fit between 0.5 and 4, indicated that the combination of germacrone and oseltamivir exerts an additive effect on the inhibition of influenza virus infection *in vitro*.

The combination study in mice was conducted using 100 mg/kg germacrone and 1 mg/kg oseltamivir, which, when used individually, protect approximately 50% of mice against influenza virus.

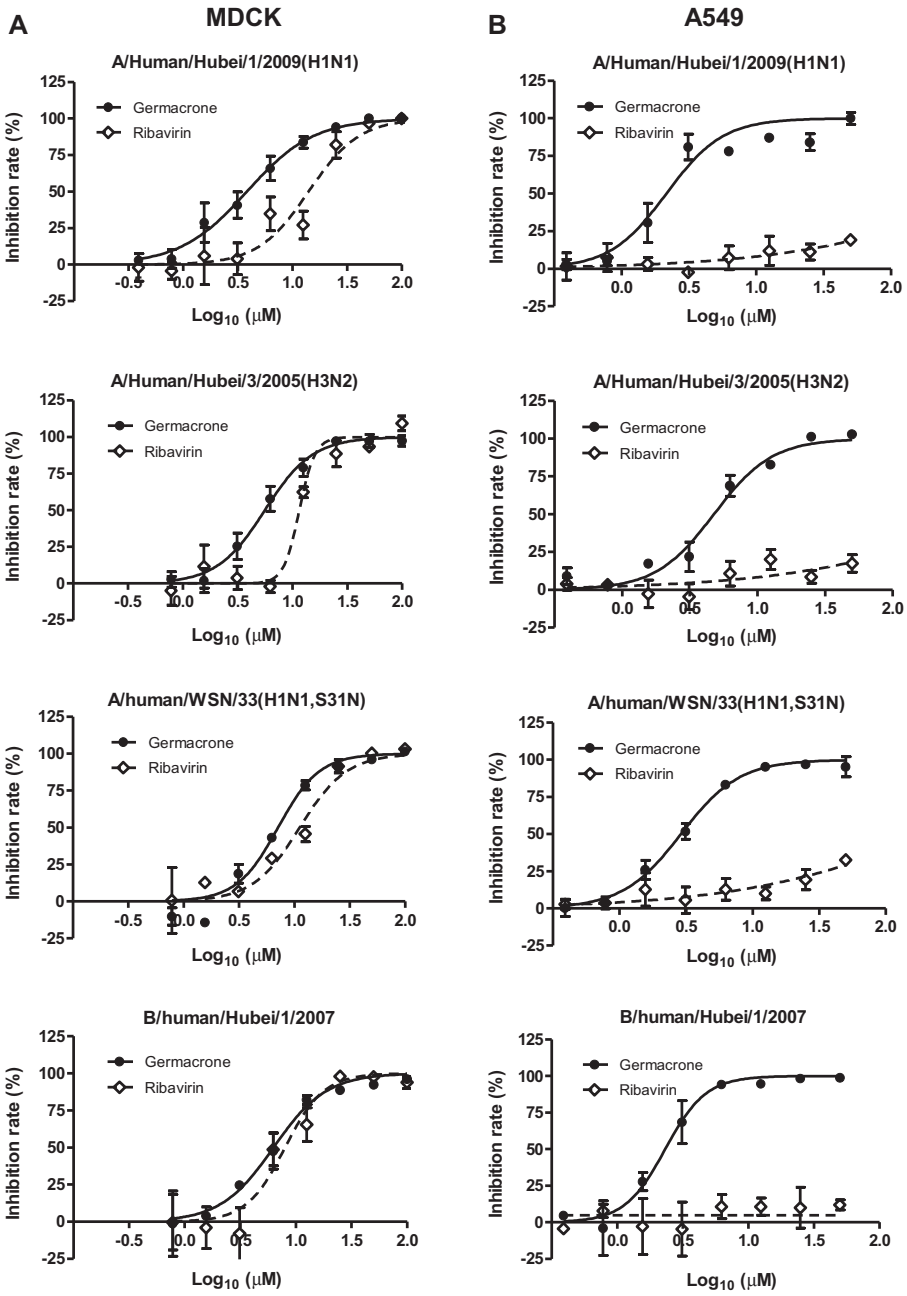

Fig. 4. Antiviral activity of germacrone against different strains of influenza virus. MDCK cells (A) and A549 cells (B) were inoculated with one of the following influenza virus strains: A/human/Hubei/1/2009(H1N1), A/human/Hubei/3/2005(H3N2), A/human/WSN/33(H1N1, S31N, amantadine resistant) and B/human/Hubei/1/2007. After 1 h at 37 °C, the cells were washed with PBS and various concentrations of germacrone (closed circle) or ribavirin (open diamond) were added. At 48 hpi, the neuraminidase activity in the supernatants was measured. The bar indicates the SD of three different experiments.

As shown in Fig. 6C, 90% survival was obtained when germacrone and oseltamivir were administered together, whereas 50% and 40% survival rates were obtained when these drugs were used alone. The results (Fig. 6D) of the virus titres of the BALF also showed that the combination of germacrone and oseltamivir exerts a significant decrease in the virus titres, whereas slightly decreases were observed when these were used alone. These results suggest that the combination of germacrone and oseltamivir exerts additive effect on the inhibition of influenza virus infection in mice.

Taken together, both the *in vitro* and the *in vivo* results showed that germacrone can efficiently be used in influenza combination therapy.

4. Discussion

Germacrone is one of the main active ingredients in the essential oils extracted from Rhizoma Curcuma, which is widely prescribed in traditional Chinese medicine for anti-tumour therapy, the promotion of blood circulation and the treatment of blood stasis. Recent studies have determined that the main bioactive constituents of Rhizoma Curcuma are its essential oils, which possess anti-inflammatory (Makabe et al., 2006), anti-tumour (Li et al., 2009), neuroprotective (Dohare et al., 2008) and vasodilator (Barrero et al., 2008) properties. A study conducted by Claeson et al. (1996) indicated that germacrone exerts significant

Table 1
Antiviral activity and cytotoxicity of germacrone against different strains of influenza viruses.

Cell line	Compound	EC_{50} (μM)[a]					CC_{50} (μM)[b]	SI[c]
		A/PR/8/34(H1N1)	A/human/Hubei/1/2009(H1N1)	A/human/Hubei/3/2005(H3N2)	Influenza B virus	A/WSN/33/S31N(H1N1)		
MDCK	Germacrone	6.03 ± 0.2	5.59 ± 0.17	3.82 ± 0.11	6.30 ± 0.19	7.12 ± 0.57	>250	>41
	Ribavirin	10.83 ± 0.19	14.03 ± 0.49	11.56 ± 1.88	8 ± 0.73	11.17 ± 0.4	137	12.6
	Oseltamivir	0.026 ± 0.001	N/A	N/A	N/A	N/A	>250	>9615
A549	Germacrone	2.66 ± 0.66	2.9 ± 0.15	2.15 ± 0.57	2.33 ± 0.37	4.78 ± 0.33	>250	>93.9

[a] The EC_{50}s were determined using the neuraminidase activity and are presented as the mean ± SD (n = 3) as described in Section 2.4.
[b] The CC_{50}s were determined by an MTT assay and are presented as the mean ± SD (n = 3) as described in Section 2.3.
[c] The SI represents the ratio of CC_{50} to EC_{50} for A/PR/8/34 strain.

anti-inflammatory activity against carrageenan-induced hind paw oedema in rats. Matsuda et al. (1998) reported that germacrone exhibits a potent protective effect on D-GalN/LPS-induced and tumour necrosis factor-alpha (TNF-)-induced acute liver injury in mice. Another study (Zhong et al., 2011) demonstrated that germacrone inhibits the proliferation of breast cancer cells by inducing cell cycle arrest and apoptosis. However, studies on the antiviral activity of germacrone have not yet been reported. To the best of our knowledge, this study is the first demonstration of the anti-influenza virus activity of germacrone. The results from this study show that germacrone inhibits the H1N1 and H3N2 influenza A viruses and the influenza B virus *in vitro* in both MDCK cells and A549 cells. We further confirmed that germacrone can reduce influenza virus RNA transcription, protein expression and progeny virus production in a dose-dependent manner in cell cultures. The reduction in the transcription of viral mRNAs, the translation of viral proteins and the production of infectious progeny viruses mediated by germacrone may not be the direct antiviral targets. Instead, these observations might be the result of the inhibition of cellular targets and early events in the viral lifecycle. Taken together, our results verified that germacrone inhibits influenza virus replication *in vitro*.

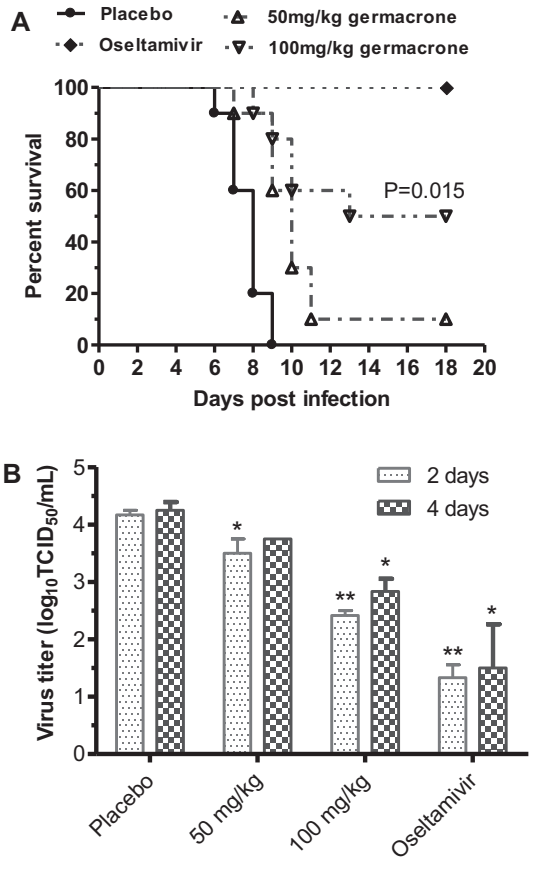

Fig. 5. Efficacy of germacrone against influenza virus *in vivo*. Sixteen mice per group were intranasally infected with 5 LD_{50} of the A/PuertoRico/8/34 viruses. The infected mice were intravenously administered germacrone or orally administered oseltamivir daily at the indicated doses for 6 days, beginning 1 day before infection. (A) The mice morbidity was observed daily throughout an 18 day observation period. (B) On days 2 and 4 post infection, three mice from each group were sacrificed. Their lungs were harvested and the viral titres in the BALF were determined through $TCID_{50}$ analysis on MDCK cells. The results represent the means of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$ (The P values refer to the comparison between the germacrone-treated group and the placebo group).

To understand the mechanisms involved in the antiviral effect, a time-of-addition assay and an inhibition time course assay were performed to determine the effect of varying the time of germacrone addition on the replication of the influenza virus. A significant inhibition of virus replication, as represented by neuraminidase and NP expression, was observed when germacrone was added to the cells within the time frame of −2 to 4 h of viral infection. Germacrone did not exert antiviral activity when it was added after 4 h of infection, which indicates that germacrone is ineffective during the late stages, i.e., assembly and release, of the viral lifecycle. The preincubation of the host cells with germacrone also inhibits influenza virus infection. These findings indicate that germacrone is different from ribavirin, which inhibits influenza virus replication if added within the time frame of 0–4 h to MDCK cells and exerts no effect in A549 cells. Our results suggest that, in addition to the inhibitory activity against viral attachment/entry to host cells, the antiviral activity of germacrone is associated with the early steps (e.g., viral protein expression and viral RNA replication) in the influenza virus lifecycle. The importance of our findings is that the antiviral mechanism of action of germacrone differs from those of the currently licensed M2 ion channel blockers and neuraminidase inhibitors, which inhibit only one viral target. In contrast, germacrone can inhibit multiple steps in the early stages of the influenza virus lifecycle.

Germacrone exhibited a significant mortality protection of mice against influenza virus infection, although the survival is only 50% even when the mice were administered a higher concentration of germacrone. However, the infected mice that were administered germacrone showed a significantly delay in mortality. The virus titres of the BALF from mice administered germacrone also exhibited a significant decrease. Furthermore, the combination of germacrone and oseltamivir showed an additive effect on the inhibition of influenza virus infection both *in vitro* and *in vivo*. Our results suggest that germacrone may have the potential to be developed as a therapeutic agent alone or in combination with other agents for the treatment of influenza virus infection.

The influenza virus hemagglutinin (HA) protein attaches to cell receptors and plays an important role in the release of viral RNA into the cell, which causes the fusion of the viral and cellular

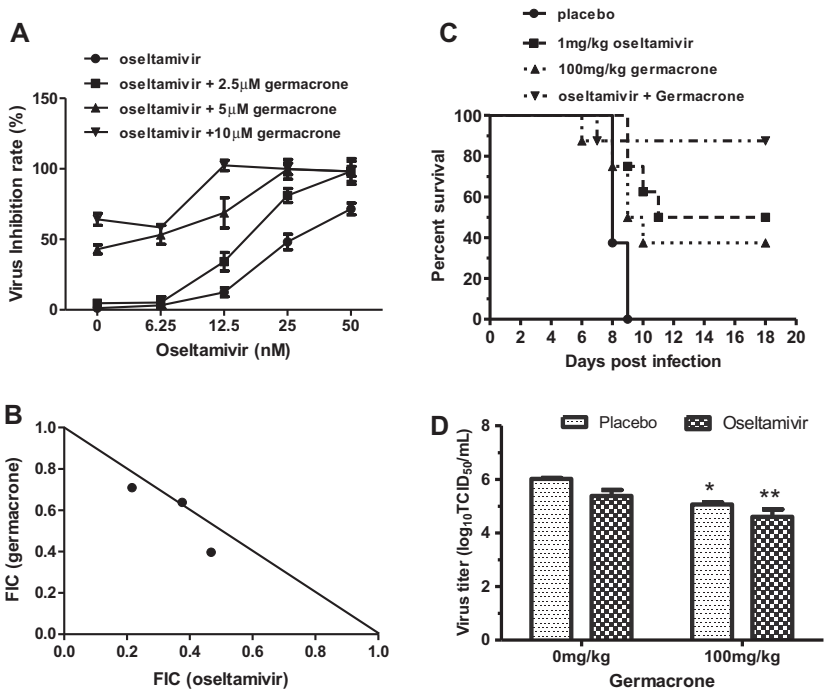

Fig. 6. Inhibition of influenza virus replication by the combination of germacrone and oseltamivir both *in vitro* and *in vivo*. Cells were treated with different concentrations of germacrone and oseltamivir either alone or in combination at the same time of virus infection. The inhibition of viral replication was measured through the MTT assay. (A) The dose-dependent curves of the combination of germacrone and oseltamivir on influenza virus replication are shown. A representative experiment from three independent repetitions (showing the standard deviation of the mean) is shown. (B) The EC_{50} isobologram of the combination of germacrone and oseltamivir is shown. The oblique line indicates the expected additive line. The calculated combination effects, which are shown by the closed circle, are all located near the additive line. The FIC indexes fluctuated by approximately 1, which is between the range of 0.5–4. This finding indicates the additive inhibition of influenza virus replication by the combination of oseltamivir and germacrone. (C and D) Thirteen mice per group were intranasally infected with 5 LD_{50} of the A/PuertoRico/8/34 viruses. The infected mice were administered germacrone alone or together with oseltamivir daily at the indicated doses for 6 days, beginning 1 day before infection. The mice morbidity was assessed daily throughout an 18 day observation period (C). On day 4 post infection, three mice from each group were sacrificed. Their lungs were harvested and the viral titres in the BALF were determined by $TCID_{50}$ analysis on MDCK cells (D). The results represent the means of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$. (The *P* values refer to the comparison between the compounds-treated group and the placebo group).

membranes. HA must be cleaved by cellular proteases to become activated as a fusion protein (Chaipan et al., 2009). The influenza virus neuraminidase (NA) is reported to be important for the initiation of influenza virus infection (Matrosovich et al., 2004). We tested whether germacrone impaired the functions of HA or NA and the results showed that germacrone does not interfere with either the hemagglutination activity of HA or the cleavage of the glycosidic linkages of the neuraminic acids of NA (data not shown). We also tested the inhibitory effects of germacrone on virus-induced CPE in HIV infected MT-4 cells and on HBV HBeAg and HBsAg expression in HepG2.117 cells and no antiviral activity was detected (Wan et al., 2012 and unpublished data), which indicates that the antiviral activity of germacrone may be specific to influenza virus. Further experiments should be performed to clarify the exact mechanism of action of germacrone on viral lifecycles.

It is anticipated that this research will establish a proof-of-concept for the development of a new class of inhibitors of influenza viruses. New efforts may be placed on the study of the antiviral mechanism of action and on the synthesis and screening of germacrone derivatives that may be more potent inhibitors of influenza viruses and less toxic than germacrone. Furthermore, because Rhizoma Curcuma has been shown to inhibit influenza viruses *in vitro* and *in vivo*, a new formula of traditional Chinese medicine could be developed for the treatment of influenza virus infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.09.021.

References

Barrero, A.F., Herrador, M.M., Arteaga, P., Catalan, J.V., 2008. Germacrone: occurrence, synthesis, chemical transformations and biological properties. Nat. Prod. Commun. 3, 567–576.
Berenbaum, M.C., 1989. What is synergy? Pharmacol. Rev. 41, 93–141.
Chaipan, C., Kobasa, D., Bertram, S., Glowacka, I., Steffen, I., Tsegaye, T.S., Takeda, M., Bugge, T.H., Kim, S., Park, Y., Marzi, A., Pohlmann, S., 2009. Proteolytic activation of the 1918 influenza virus hemagglutinin. J. Virol. 83, 3200–3211.
Claeson, P., Pongprayoon, U., Sematong, T., Tuchinada, P., Reutrakul, V., Soontornsaratune, P., Taylor, W.C., 1996. Non-phenolic linear diarylheptanoids from curcuma xanthorrhiza: a novel type of topical anti-inflammatory agents: structure-activity relationship. Planta. Med. 62, 236–240.
De Clercq, E., 2004. Antivirals and antiviral strategies. Nat. Rev. Microbiol. 2, 704–720.
De Clercq, E., 2006. Antiviral agents active against influenza A viruses. Nat. Rev. Drug. Discov. 5, 1015–1025.
Dohare, P., Garg, P., Sharma, U., Jagannathan, N.R., Ray, M., 2008. Neuroprotective efficacy and therapeutic window of curcuma oil: in rat embolic stroke model. BMC Complement. Altern. Med. 8, 55.
Furuta, Y., Takahashi, K., Kuno-Maekawa, M., Sangawa, H., Uehara, S., Kozaki, K., Nomura, N., Egawa, H., Shiraki, K., 2005. Mechanism of action of T-705 against influenza virus. Antimicrob. Agents. Chemother. 49, 981–986.
Kamps, B.S., Hoffmann, C., Preiser, W., 2006. Influenza Report 2006, 225.
Li, Y., Wo, J.M., Liu, Q., Li, X., Martin, R.C., 2009. Chemoprotective effects of curcuma aromatica on esophageal carcinogenesis. Ann. Surg. Oncol. 16, 515–523.
Makabe, H., Maru, N., Kuwabara, A., Kamo, T., Hirota, M., 2006. Anti-inflammatory sesquiterpenes from curcuma zedoaria. Nat. Prod. Res. 20, 680–685.

Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J. Virol. 78, 12665–12667.
Matsuda, H., Ninomiya, K., Morikawa, T., Yoshikawa, M., 1998. Inhibitory effect and action mechanism of sesquiterpenes from zedoariae rhizoma on D-galactosamine/lipopolysaccharide-induced liver injury. Bioorg. Med. Chem. Lett. 8, 339–344.
McKimm-Breschkin, J.L., 2000. Resistance of influenza viruses to neuraminidase inhibitors – a review. Antiviral. Res. 47, 1–17.
Molinari, N.A., Ortega-Sanchez, I.R., Messonnier, M.L., Thompson, W.W., Wortley, P.M., Weintraub, E., Bridges, C.B., 2007. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine 25, 5086–5096.
Moscona, A., 2005. Neuraminidase inhibitors for influenza. N. Engl. J. Med. 353, 1363–1373.
Odds, F.C., 2003. Synergy, antagonism and what the chequerboard puts between them. J. Antimicrob. Chemother. 52, 1.
Peiris, J.S., Poon, L.L., Guan, Y., 2009. Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. J. Clin. Virol. 45, 169–173.
Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69, 517–528.
Rajik, M., Jahanshiri, F., Omar, A.R., Ideris, A., Hassan, S.S., Yusoff, K., 2009. Identification and characterisation of a novel antiviral peptide against avian influenza virus H9N2. Virol. J. 6, 74.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpionts. Am. J. Epidemiol. 27, 493–497.
Rothberg, M.B., Haessler, S.D., Brown, R.B., 2008. Complications of viral influenza. Am. J. Med. 121, 258–264.
Song, J.M., Lee, K.H., Seong, B.L., 2005. Antiviral effect of catechins in green tea on influenza virus. Antiviral. Res. 68, 66–74.
Wan, Z., Lu, Y., Liao, Q., Wu, Y., Chen, X., 2012. Fangchinoline inhibits human immunodeficiency virus type 1 replication by interfering with gp160 proteolytic processing. PLoS One 7, e39225.
Weinstock, D.M., Zuccotti, G., 2006. Adamantane resistance in influenza A. JAMA 295, 934–936.
Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., McKimm-Breschkin, J.L., Zambon, M., Hayden, F.G., 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J. Clin. Microbiol. 41, 742–750.
Zhong, Z., Chen, X., Tan, W., Xu, Z., Zhou, K., Wu, T., Cui, L., Wang, Y., 2011. Germacrone inhibits the proliferation of breast cancer cell lines by inducing cell cycle arrest and promoting apoptosis. Eur. J. Pharmacol. 667, 50–55.