Identification of Antiviral Activity of Koe-Ken Tang Against Influenza Virus

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Abbreviations: KKT, Koe-Ken Tang; pfu, plaque forming unit; PI3K, phosphatidylinositol 3-Kinase; moi, multiplicity of infection; p.i., post viral infection; RNP, ribonucleoprotein;

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ABSTRACT

Ethnopharmacological relevance: Ko-Ken Tang (KKT, aka kakkon-to), a conventional Chinese herbal medicine, has been used for the treatment of common cold, fever and influenza virus infection. However, the underlying mechanism against influenza virus infection still remains elusive.

Aim of study: To investigate the functional mechanism of KKT against influenza virus infection.

Materials and methods: The anti-influenza virus activity and cytotoxicity effect of KKT on Madin-Darby canine kidney cells was determined. Mode of action and inhibition of viral polymerase activity was measured by time-of-addition assay and by primer extension assay, respectively. Inhibition of virus-induced phosphatidylinositol 3-kinase (PI3K)/Akt pathway by KKT was analyzed by western blotting. Inhibition of nuclear export of viral ribonucleoprotein complex by KKT was determined by indirect immunofluorescence microscopy and heterokaryon assay.

Results: These data demonstrate that KKT inhibited influenza virus at the replication but not entry stage and it exhibits a broad inhibition spectrum against human influenza viruses A. KKT not only blocked virus-induced Akt phosphorylation which causes retention of viral nucleoprotein (NP) in the nucleus to interfere with virus propagation.
Conclusions: KKT could be a potential use on the management of seasonal pandemic influenza virus infection as well as other available clinical drugs.

Key words: cytopathic effect, influenza virus, kakkon-to, Ko-Ken Tang, nucleoprotein, phosphatidylinositol 3-kinase/Akt
1. Introduction

Influenza still remains the major plagues as well as the highly pandemic outbreak in the world. Influenza virus is an enveloped RNA virus, belonging to the family Orthomyxoviridae (Palese et al., 2007). The genome characteristic of influenza A virus was an eight-segmented negative polarity single stranded RNA and the reassortment of different influenza virus strains contribute to both seasonal epidemics and sporadic pandemics. The pandemic H1N1 influenza was a typical example of viral gene reshuffle between human, swine, and Eurasian avian strains. In concern of antiviral drugs, such as viral M2 ion channel inhibitor and viral neuraminidase inhibitor which have been provided helpful prognosis for the treatment of influenza virus infection, has started to come out of resistant virus in many clinical cases due to this highly mutational RNA-dependent RNA polymerase fidelity (Hauge et al., 2009; Meijer et al., 2009; de Jong et al., 2005; Le et al., 2005; Hayden et al., 2005). A variety of investigations have been looking for the alternative drugs to treat emerging mutants.

In addition, influenza virus makes use of the cellular machinery to replicate, thus blocking of cellular mechanisms required for viral replication may be an alternative approach to inhibit virus growth. Upon influenza virus infection, various cell signaling alterations have been determined, such as Raf/MEk/ERK, nuclear
factor(NF)-κB and PI3K/Akt, etc (Stephan et al., 2006). In several studies, PI3k had been addressed for its role to modulate anti-apoptosis and cytokine production (Ehrardt et al., 2007). Moreover, it was initially described to act as an antiviral fashion (Sarkar et al., 2004), but latter it was demonstrated that the activation of PI3K/Akt signaling induced by influenza A virus supports efficient replication of viruses (Ehrardt et al., 2006) and the viral non-structural protein 1 (NS1) take part in this activation (Hale et al., 2006).

Ko-Ken Tang, a traditional Chinese prescription, has been commonly used in the treatment of HSV-1 infection (Nagasaka et al., 1995), fever (Masahiko et al., 1998), colds, coughs, and flu in the Asian countries. Furthermore, KKT was showed effectively against influenza virus infection in in vivo model (Kurokawa et al., 2002), though without elucidating its molecular antiviral mechanisms. In this regard, we have documented its anti-influenza virus efficacy in cell-based model and dissected its mode of action underlying viral infection. In this report, we demonstrated that KKT could down-regulate the activation of PI3K/Akt signaling cascade induced by influenza A virus and thus interfering the propagation of virus. Therefore, KKT is presumed to be a consecutive anti-influenza virus prescription even in the future influenza infection therapy.
2. Materials and methods

2.3 Cell culture, viruses, and reagents

MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS), 2mM L-glutamine and 2 mM NEAA. Human embryonic kidney (293T) cells were cultivated in DMEM medium containing 10% FBS. Human lung carcinoma (A549) cells were cultivated in minimal essential medium (Invitrogen) supplemented with 10% FCS, and 2mM NEAA. Influenza A/WSN/33 (H1N1) virus stocks were utilized for this study and viral titers were determined with stand plaque assay. Unless stated otherwise, WSN were used in all experiments. PI3K/Akt specific inhibitor LY294002 was also used in whole study as a control. Enterovirus 71 which belongs to Picornavirus family was used in the neutralization assay.

2.2 Preparation of crude herb extract

Ko-Ken Tang (KKT) is composed of seven medicinal herbs which are Pueraria root (*Puerariae Radix*), Ma huang (*Ephedrae Herba*), Cinnamon twigs (*Cinnamomi Ramulus*), Peony root (*Paeoniae Lactiflorae Radix*), Jujube date (*Zizyphi Sativae Fructus*), Licorice (*Glycyrrhizae Radix*) and Ginger (*Zingiberis Siccatum Rhizoma*) as described previously (Nagasaka et al., 1995; Kurokawa et al., 1996). KKT was purchased from Sun Ten pharmaceutical Co., Ltd., (Taiwan). Water extract of KKT
was prepared as reported before (Yen et al., 1991) with a minor modification. Instead of hot water extraction, KKT was immersed in distilled water and incubated at 37°C water bath for 16 h. Supernatant was initially collected after 1,500 rpm 10 min sedimentation at 10°C, and subsequently subjected to 15,000 rpm for another 30 min. Finally, aqueous extract was obtained with 0.22 μm Nalgene filtration. The extract of KKT was diluted in sterile DMEM medium to final concentrations.

2.3 Cytotoxicity assay, antiviral effectiveness assay and selectivity index (SI)

The cytotoxicity effect of KKT on Madin-Darby canine kidney (MDCK) cells was determined with MTT-based assay and crystal violet staining method, respectively (7). MDCK cells (2 x 10⁴/well) were seeded into 96-well culture plates for overnight incubation. The medium was removed and cells were incubated with various concentrations of KKT medium without serum for 72 h. The medium was removed and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added at a concentration of 5 mg/ml and incubated for 3 h at 37°C. Then 50% cell cytotoxic concentration (CC₅₀) of KKT was calculated according to Reed–Muench method. Besides, the anti-influenza virus activity of KKT was also assayed by crystal violet staining method. Briefly, after cell seeding for 24 h, medium was removed and cells were infected with influenza virus of 9 TCID₅₀, then various concentrations of KKT were supplemented immediately for incubation of another 72 h. Then the medium was
aspirated and the cyto-protective activity of KKT was determined by crystal violet staining. The visual staining results were determined with a microplate reader (Perkin Elmer, VICTOR³ multilabel Readers). Each experiment was determined in triplicate and performed at least thrice separately. The antiviral activity of KKT with its minimal concentration required to inhibit 50% cytopathic effect of influenza virus (IC₅₀) was estimated.

2.4 Time of addition assay and multicycle growth inhibition assay

MDCK cells were seeded onto 6-well plates at a density of 5 x 10⁵ cells per well and incubated at 37 °C with 5% CO₂ for 16 h. The cell monolayer was infected with influenza virus WSN at a multiplicity of infection (moi) of 1 plaque forming unit (pfu) per cell for 1 h at 4 °C. KKT was added to cells either at -3 h (2 h before viral infection), 0 h (during viral infection), or 1 h p.i. (post viral infection, Fig. 1). At 10 h p.i., supernatant was collected and virus yields were subsequently determined by plaque assay. For multicycle experiments, MDCK cells were infected with virus of 0.01 M.O.I at 37°C for 1 h. The virus-infected MDCK cells were either incubated with 4 μg/μl KKT or DMEM only, and supernatant was collected at 8, 16, 24 h p.i. for viral titer determination. Briefly, monolayer MDCK cells (6 x 10⁵ cells/well) were washed once with Dulbecco’s phosphate-buffered saline (DPBS), and infected with
serially diluted viral suspension. After adsorption for 1h at 37°C, the viral suspension was replaced with overlay medium, E₀ (DEME with L-glutamine (2mM), and nonessential amino acid mixture (0.1mM) containing 2.5 mg/ml trypsin and 0.3% agarose. After incubation for 2-3 days at 37°C under 5% CO₂, the cells were fixed with 4% paraformaldehyde and then stained with 1% crystal violet. The titer of the virus was expressed in plaque-forming units (PFU) per milliliter.

2.5 RNP activity measured by reporter assay

Monolayers of HEK293 at 5 x 10⁴ cells/well were seeded onto 48-well culture plates and incubated overnight. NP, PA, PB1, and PB2 in pHW2000 expression vectors (0.1 μg each) plus pPOLI-Fluc plasmid (0.1 μg) and pRL-TK (5 ng, Promega) were co-transfected into HEK293 using calcium chloride and incubated for another 8 h. After removing medium, varying concentrations of KKT were added into wells and incubated for 24 h. Cells were then harvested for luciferase assay using Dual-luciferase Assay System (Promega, Madison, WI) and an LMax II 384 (Molecular Device). Values at each dose were analyzed in triplicate and graphed with standard deviations. This is an average result from three experiments and the y-axis is the ratio of Fluc to Rluc controls, normalized to mock treatment (set arbitrarily to 1.0).

2.6 RNP activity measured by primer extension assay

Primer extension reactions were performed using a primer extension system—the
AMV reverse transcriptase kit (Promega)—following the manufacturer’s instructions.

One μg of each pHW2000-PB1, PB2, PA, and NP and pPOLI-CAT-RT were
cotransfected into 293 cells in a 6-well plate (Pleschka et al., 1996). KKT was added
after 8 h p.i. and incubated for further 24 h. Total RNA was extracted with Trizol
reagent (Invitrogen). Briefly, 5 μg of total RNA was mixed with CAT vRNA-specific
\(^{32}\text{P}\)-labelled primer and positive-sense RNA-specific \(^{32}\text{P}\)-labelled primer,
ATGTCTTTTACGATGCGATTGGG and negative-sense RNA-specific \(^{32}\text{P}\)-labelled
primer, CGCAAGGCGACAAGGTGCTGA. Primer extensions were performed at 42
\(\text{o}\)C for 2 h. Transcription products were denatured at 90 \(\text{o}\)C for 10 min, separated on
8% polyacrylamide gels that contained 7 M urea in Tris/borate/EDTA (TBE buffer)
buffer and detected by autoradiography.

2.7 Viral infection, solubilization, electrophoresis, and Western blot analysis

Cells were grown to 70-80% confluence in complete medium, where upon the
cells were serum starved by incubation in serum-free growth medium (E\(_0\)) for 24h. For
viral infection, growth arrested cells were mock-infected or infected with influenza
virus at an moi of 1 for 1h. Cells were washed with HBSS and then cultured in fresh
medium with or without inhibitor for various length of time as indicated during the
course of infection. Cells were washed twice with PBS and lysed with lysis buffer
(150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH
7.5, and 2 mM EDTA) with freshly added protease inhibitors. After incubation for 15 min on ice, cell lysate was centrifuged at 14,000 xg for 10 min at 4 °C to remove insoluble cell debris. Protein concentration was determined with Bio-Rad protein assay kit. Equal amount (50 μg) protein was subjected to 10% SDS-PAGE (polyacrylamide gels) then transfer onto a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated overnight at 4 °C with polyclonal antisera for Akt, phosphor-Akt(Ser473) (Cell Signaling), mouse monoclonal influenza virus nucleoprotein (NP) (Abcam, # ab20343), M1 (matrix protein 1) goat anti-influenza A virus M1 (ViroStat, #1321), or GAPDH antibody (Santa Cruz). After incubation with antibodies for indicated times, membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary anti-species antibodies, followed by visualization of positive bands by using an enhanced chemiluminescence western blotting detection system (Millipore, Billerica, MA, USA). Protein bands were scanned and intensity was calculated with Quantity One program (Bio-Rad), version 4.6.2.

2.8 Indirect immunofluorescence microscopy

MDCK cells (1 x 10^5) were plated onto coverslips 1 day before infection. The cells were infected with influenza virus for 1 h on ice, then washed twice with HBSS to remove unattached virus. DMEM medium with or without KKT was added and
subsequently incubated at 37°C for 9 h p.i.. The following procedures were performed
at room temperature unless otherwise indicated. Cells were fixed with 4%
paraformaldehyde (PFA) in PBS for 20 min followed by permeabilized with 0.5%
Triton X-100 in PBS for 5 min. After blocking in blocking buffer (0.5% BSA in PBS)
for 1 h, cells were incubated with primary and secondary antibodies in blocking buffer
for 1 h, respectively and then followed by washing with blocking buffer three times
and stained with DAPI for 10 min. Finally, cells were mounted in mounting medium
(Dako) and viewed by Zeiss Axiosvert 200M fluorescence microscope fitted with 100x
oil immersion objective lens.
3. Results

3.1 Antiviral efficacy and cytotoxicity assay

Selected Chinese herbal mixtures to treat respiratory diseases were screened for their capacity to inhibit influenza-virus-induced cell death in a neutralization assay. Influenza virus A/WSN/33 was used to infect MDCK cells in the presence or absence of herbal mixtures and a reduction in virus-induced cell death indicated antiviral activity, in which zanamivir (Relenza) was used as the positive control. KKT was one of several initial hits displaying an IC\textsubscript{50} of less than 2 μg/μl, with no apparent cytotoxicity (Table 1). KKT was effective against WSN induced cytopathic effect with IC\textsubscript{50} of 1.15 μg/μl and the 50% cytotoxic concentration (IC\textsubscript{50}) of KKT was 5.5 μg/μl by MTT assay using MDCK cells. Furthermore, according to plaque reduction assay, 4 μg/μl KKT (this is near CC50) was the minimum concentration to completely inhibit plaque formation, thus this concentration was chosen for the latter experiments. We also test the inhibition spectrum of KKT against other human influenza viruses (Table 1). KKT inhibited not only the laboratory-adapted WSN strain with long passage history but also clinical strains isolated after 1998. Oseltamivir-resistant seasonal influenza became prevalent in north hemisphere from late 2007 (Hauge et al., 2009; Meijer et al., 2009; Baranovich et al., 2010). Three clinical resistant seasonal influenza viruses (H1N1) isolated locally in 2009 were examined for their
susceptibility to KKT. Except one strain, KKT has similar potency to the other two resistant strains (Table 1).

KKT is composed of seven herbal components which are pueraria root, ma huang, cinnamon twigs, peony root, jujube date, licorice and ginger (Nagasaka et al., 1995; Kurokawa et al., 1996). These components were purchased from the same supplier as KKT and were tested for their anti-influenza virus activity using neutralization assay. Table 2 shows the antiviral activity of each herb from KKT. Among these, only ma huang and cinnamon twigs were found to have antiviral activity in which the former has better potency than KKT itself (IC50 0.44 ± 0.15 μg/μl). These data imply that ma huang and cinnamon twigs are the major bioactive herbs of KKT and the rest may play supportive roles.

3.2 Time of addition assay in influenza A virus-infected cells

The underlying antiviral mechanism of KKT was first determined by time-of-addition assay in a single infectious cycle. Effectiveness of KKT in reducing the viral yield with regard to the time in which KKT was added to the culture medium during the course of infection (Figure 1). The treatment protocol of KKT was illustrated in Fig. 1A and arrows indicate the presence of KKT before, during (-1 to 0 h p.i.) or after virus infection. The supernatants from cells treated with KKT or virus only were
harvested for plaque assay at 10h p.i. (Fig. 1B). Neither did pre-treatment of the cells with KKT before (-3 to -1 h p.i.) and during (-1 to 0 h p.i.) virus adsorption and removal of it subsequently, nor early treatment (0 to 1 h p.i.) showed any protection effect. A notable and significant reduction was observed when KKT was applied after viral adsorption (1h after viral infection), in which titers of progeny virus were reduce approximately by 100-fold (Fig. 1B). It implied that KKT targets at the viral replication stage rather than at entry or adsorption.

3.3 KKT reduced PI3K activation induced by influenza A/WSN/33 infection

Next, we turned our attention to see if KKT acts at cellular proteins by determining its effect on PI3K/Akt pathway. Based on previously study, PI3K/Akt signaling pathway was activated at viral propagation stage of viral infection which is similar to our time-of-addition data (Shin et al., 2007). PI3K/Akt signaling pathway has attracted much attention recently due to its role associated with many respects of cellular functions. An increased number of documentation demonstrated that influenza virus infection activated PI3K/Akt pathway to support its replication (see review in Ehrhardt and Lugwig., 2009). We first address the specific phosphorylation of Akt at serine 473 (Ser473), a site that is targeted in a strictly PI3K-dependent manner (Alessi et al., 1996). Upon infection of A549 cells with the WSN virus, phosphorylation of
Akt became prominent as early as 3h and persisted for the rest of infection course (Fig. 2, lane 3, 9, 16 and 22). As expected, by using an antibody that detected the total Akt protein, we found that the protein levels remained unchanged following WSN infection, which is consistent with previous reports (Shin et al., 2007; Ehrhardt et al., 2007a). The equal intensity of total Akt throughout the infection, suggesting that the changes of Akt phosphorylation was not resulted from altered protein expression. The activation of Akt phosphorylation was neither due to the drug alone (lanes 4, 10, 17 and 23) nor in mock-infected cells (lanes 2, 8, 15, and 21), indicating the increase of Akt phosphorylation by viral infection was specific. Akt phosphorylation was activated in PI3K-dependent pathway because LY294002, a specific PI3K inhibitor, abrogated influenza virus induced Akt phosphorylation (lanes 6, 12, 19 and 25) (Filippa et al., 1999; kandel and Hay, et al., 1999). KKT had a similar inhibition kinetic to LY294002 which inhibited virus-induced Akt phosphorylation as early as at 3 h p.i. and the inhibition was persistent throughout the infection course (lanes 5, 11, 18 and 24). As Akt phosphorylation was needed for viral propagation and thus inhibition of virus-induced Akt phosphorylation may reduce the viral protein synthesis (Shin et al., 2007). The accumulation of viral protein was indeed inhibited by KKT in a single or multiple cycle of infection (Fig. 2A and 3B). inhibition of influenza virus by KKT via unidentified pathways may also result in reduced viral protein synthesis.
and thus reciprocally inhibit phosphorylation of Akt. We can’t exclude the possibility that KKT may target at another pathway that reduced the synthesis of viral protein and in turn inhibit PI3K/Akt pathway. We thus address whether Akt is a direct target of KKT by examining whether KKT can inhibit Akt phosphorylation in response to specific inducers. Serum has been demonstrated to serve as an mitogen and induce a marked increase of Akt phosphorylation that was inhibited completely by LY294002 (Pan et al., 2003). Akt phosphorylation was induced by serum treatment with or without LY294002 or KKT pretreatment (Fig. 2B). Phosphorylation of Akt was induced by serum treatment but was abolished by LY294002 as well as KKT, demonstration that KKT targets Akt signaling pathway directly. This indicates that inhibition of virus replication by KKT may result from the blockade of PI3K/Akt pathway as suggested previously in which PI3K/Akt signaling pathway plays a role in influenza virus replication (Shin et al., 2007; Ehrhardt et al., 2007a).

3.4 KKT impaired viral propagation and viral protein synthesis

We next investigated the role of KKT in regulating propagation as activated PI3K/Akt pathway is involved in influenza virus replication. We first examined whether inhibition of PI3K/Akt activation by KKT would change the production of influenza virus.
Although KKT was found to exert its anti-viral activity when applied at an early time after viral infection within one life cycle, we also determined the long term inhibition effect of KKT on viral propagation. As KKT was maintained in the medium, virus progeny titer at 24 h was reduced to about 100-fold lower plaques numbers while still about 10-fold less at 36 h p.i. (Fig. 3).

3.5 Viral protein synthesis is blocked upon KKT treatment

The consequences of PI3K/Akt pathway inactivation on viral protein synthesis were examined with KKT. MDCK cells infected with WSN (m.o.i. of 0.01 pfu per cell) in the presence of KKT or not were harvested at predetermined time points and lysate were subjected to Western blotting using antibodies against NP and M1, respectively. Cellular GAPDH protein was included as a loading control (Fig. 4). Quantification of the density of the protein bands was performed by using Quantity One software (Bio-Rad). Viral NP and M1 protein accumulation were normalized by GAPDH level. Synthesis of both NP and M1 proteins was evident in DMEM treated cells at the indicated time points. In contrast, in KKT-treated cells, synthesis of both viral proteins was reduced.
3.6 KKT doesn’t influence the influenza virus directly

Next we try to figure out whether the reduction of viral protein synthesis was due to the inhibition of viral RNA-dependent RNA polymerase (RdRp) activity using a plasmid-based ribonucleoprotein (RNP) assay. A/PR/8 RNP complex expression plasmids, NP, PB1, PB2 and PA and the reporter plasmids, pPOL1-CAT-RT. The reporter plasmid contained a vRNA-like RNA encoding the reporter gene for chloramphenicol acetyltransferase (CAT), in the negative sense, flanked by the 5’ and 3’ noncoding regions of the NS vRNA segment of the influenza virus (Pleschka et al., 1996). KKT was added 8 h post transfection and incubated for additional 24 h and the cells were harvested for primer extension assay. As shown in Fig. 4A, KKT did not exhibit any reduction of three RNA species at concentration of up to 4 μl/μl, a concentration which can markedly inhibit viral protein synthesis and progeny virus production. This indicates that KKT did not target influenza virus replication at the RNA-dependent RNA polymerase (RdRp) activity. In addition to the primer extension assay, we further carried out reporter assay to see the effect of KKT on RNP activity through a plasmid-based reverse genetics. Increasing concentrations of KKT were incubated with HEK293 cells previously transfected with pHW2000-PB2, PB1, PA, NP, and pPOL1-firefly luciferase, together with pTK-RL as a transfection control. After treatment of KKT for 24h, cell lysates were prepared for luciferase assay. KKT
did not inhibit the activity of FLuc activity, indicating that KKT did not target viral RNP complex, which drives the reporter gene expression (Fig. 4B).

3.7 KKT treatment results in NP retention in the nucleus

As previously study showed, influenza virus transcription occurs in the nucleus of infected cells and viral RNAs must be exported in the form of RNP complex from the nucleus. RNP complex nuclear export of WSN virus in the presence of KKT and LY294002 was studied by immunofluorescence staining of the NP protein, which is the major component of the RNP complex. As visualization in Fig.6a, NP was found mainly in the cytoplasm of WSN infected cells at 9 h p.i., indicating that the majority of RNP complex had been exported from the nucleus. However, NP was accumulated in the nucleus of WSN infected and KKT treated cells (Fig. 6b), indicating that nuclear export of RNP complex is perturbed. Cells were counterstained with DAPI (Fig. 6c, d).
4. Discussion

Influenza viruses have imposed a serious health issue in morbidity and mortality. The recent emergence of NA inhibitors and M2 ion channel resistant viruses has raised the concern of new pandemic influenza strains. Thus it is crucial to search for the possible replaceable anti-influenza virus drugs. KKT is one of the representative traditional herb medicines and has been used historically for the treatment of infectious diseases in Asian countries (Nagasaka et al., 1995). The increased survival rate and inhibition of body weight loss exerted by KKT was demonstrated in influenza virus infected mice (Kurokawa et al., 2002). However, the evidence of inhibition of viral replication in cell culture system has not been reported and the mode of action of KKT thus remains illusive. Here we demonstrated that KKT was broadly effective to block cytopathic effect induced by influenza A viruses in Madin-Darby Darly canine cell line as well as oseltamivir resistant virus, albeit less effective in influenza B strains. The inhibition by KKT of the oseltamivir-resistant virus is comprehensible by considering the difference between its antiviral mechanism and that of oseltamivir. We found that KKT could exert its anti-viral activity when applied after viral infection demonstrating that it is applicable in the clinical usage. Usually, patients who have clinical syndromes would come to apply drugs for therapy rather than for prevention and the western blot data of PI3K/Akt also support our
assumption that KKT could block the virus propagation at the later stage after viral infection. On the basis of earlier findings that PI3K/Akt pathway is required for the efficient influenza virus propagation and we have identified another mechanism by which the phosphorylation of Akt was down-regulated by KKT to inhibit viral propagation. In this study, we investigated the molecular mechanisms how KKT was used to inhibit viral propagation. Here we described that KKT could block PI3K/Akt phosphorylation induced by influenza A virus besides, we also find out that KKT could block virus-induced cleavage of PARP comparing to virus infection treatment (data not shown). We deduced that KKT must still have some unknown antiviral activities rather than those drugs aimed at virus only, and it might be suitable to be a good prescription for the treatment of highly mutational influenza virus.

KKT is made of seven medicinal herbs, namely, pueraria root, ma huang, cinnamon twigs, peony root, jujube date, licorice and ginger (Nagasaka et al., 1995; Kurokawa et al., 1996b). We examined the anti-influenza virus activity for each component herb and only ma-huang and cinnamon twigs showed such activity. Ma-huang and its constituent (+)-catenin have been shown to possess anti-influenza virus activity using MDCK cells (Mantani et al., 1999; 2001). We also identified that cinnamon twigs possesses anti-influenza virus with IC50 of 1.53 ± 0.41 μg/μl where its principal ingredient cinnamaldehyde has been reported to possess anti-influenza/PR/8 activity
(Hayashi et al., 2007). The primary component of licorice, glycyrrhizin, has been evaluated experimentally in the mouse model against influenza virus. Glycyrrhizin may protect mice exposed to a lethal dose of influenza virus through the induction of interferon-gamma production by T cells (Utsunomiya et al., 1997). Glycyrrhizic acid has been shown to affect the growth of viruses in embryonic eggs at the late viral replication steps, although the viral viability and hemagglutinating activity of the virions were not impaired (Pompai et al., 1983). On contrast, we were not able to detect the anti-influenza virus activity of licorice in MDCK cells. This difference may be explained by the following. First and most likely, the discrepancy is caused by different assay systems, in which the embryonic tissues and cell cultures were employed, thus may result in different consequence. Second, the concentration of bioactive ingredients of licorice, namely, Glycyrrhizic acid and glycyrrhizin, are too low in our extract fraction. With regard to the anti-influenza bioactive components present in ma-huang and cinnamon twigs, more effort will be made to identify these components.

Influenza viruses, like other viral pathogens, hijack factors of the host cell signaling machinery for efficient replication (Ludwig et al., 2006; Ludwig, 2007). In this study, we investigated the inhibition of PI3K/Akt signaling pathway by KKT. We found that cellular Akt was phosphorylated at 3 h p.i. and was persistent throughout the rest of
the infection. Akt phosphorylation and viral protein synthesis were abolished by KKT and a PI3K-specific inhibitor, LY294002 (Fig. 2A). Without viral infection, KKT vigorously inhibited serum-induced Akt phosphorylation (Fig. 2B), demonstrating that the reduction of viral protein synthesis and replication was a result of direct inhibition of the PI3K/Akt pathway by KKT. This is consistent with the observation that Akt phosphorylation occurred in the late, rather than early, stage of infection (Fig. 1a). This also supports the idea that post-entry events are required for cellular PI3K/Akt activation, such as RNP export from the nucleus, inhibition of pre-mature apoptosis, and viral packaging (Zhirnov and Klenk, 2007). We tested the inhibitory activity of KKT against influenza polymerase in a cell-based assay and found that this compound does not inhibit influenza polymerase activity, suggesting this activity is not regulated by PI3K/Akt (Fig. 4). Interesting, KKT inhibited oseltamivir-resistant viruses, suggesting KKT did not target NA-associated functions and provide as an alternative antiviral (Table 1). We demonstrated that in cells treated with KKT, viral protein synthesis as well as nuclear export of viral RNPs were impaired which is similar to the finding by Shin et al (2007b). Although KKT is proposed to target PI3K/Akt pathway we can not exclude the possibility that KKT targets at viral proteins, such as NS1 protein of influenza A virus which is required and sufficient for activation of PI3K/Akt by binding to the regulatory subunit p85 of PI3K (Hale et al.,
We observed that KKT plays an important role at late stage of viral replication in a time-of-addition assay and did not inhibit influenza B viruses in the inhibition spectrum assay which is co-incident with the finding by Ehrhardt et al., (2007b). Although the NS1 protein of influenza A and B viruses shares less than 20% identity of protein sequence, both proteins reveal similar functions such as inhibition of the IFN-induced PKR (Talon et al., 2000; Garcia-Sastre, 2004;)

5. Conclusion

As previously shown, KKT could reduce the inflammation effects and fever syndrome post viral infection in mouse model. In this study, we further demonstrate that KKT is able to down-regulate PI3K/Akt signaling pathway induced by influenza A virus to inhibit virus propagation in cell based system. Moreover, the present data demonstrated that KKT indeed possesses promising anti-influenza A virus activities.

KKT may

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Acknowledgements

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References


Figure legends

**Fig. 1.** Time of addition assay of KKT on MDCK cells. Supernatant were collected until 10 h p.i. Viral titer was determined with stand virus plaque assay. KKT was effective when applied 1 h after viral infection. Data were presented as mean±SD of results from triplicate experiments.

**Fig. 2.** KKT inhibits influenza virus induced PI3K/Akt signaling cascade. (A) Serum-starved A549 cells were incubated with A/WSN at an moi of 1 for 1 h and then the cells were washed with HBSS and replenished with E0 containing indicated inhibitors (20 μM LY294002 or 4 μg/μl KKT) or DMSO. (B) Serum-starved A549 cells were pre-incubated with LY294002 or KKT for 5 h followed by serum (5%) treatment in the presence or absence of LY294002 or KKT for 3 h. Cell lysates were collected at the indicated times post infection and equal amounts of protein (50 μg per lane) were subjected to SDS-10% PAGE. Proteins were transferred onto PVDF membranes and subjected to Western blotting using antibodies against phospho-Akt, total Akt, or NP. Cells treated with normal growth medium (E10) served as positive controls (lanes 1 and 14 in Fig. 2A). This is a representative data from at least three independent experiments.
**Fig. 3.** KKT possessed protection activity in multiple infectious cycles. MDCK cells were adsorbed with A/WSN (moi 0.01) for 1 h and the unbound viruses were removed and replenished with E0 containing KKT (4 μg/μl). (A) Supernatants at 8 h, 16 h and 24 h p.i. were collected and diluted to assay the titers of progeny virus (plaque assay). Data were presented as mean±SD of results from three independent reproducible experiments. (B) Cell lysates prepared at the indicated time point were subjected to western blotting using antibody against NP, M1, and GAPDH. This is a representative result from two independent reproducible experiments. (C) KKT inhibits virus-induced CPE. MDCK cells were infected with A/WSN/33 virus or mock infection and antiviral effect of KKT was subsequently evaluated morphologically 30 h p.i. under a light microscope. a. MDCK cells were mock infection; b. cells were infected with virus; c. cells were only incubated with KKT; d. cells were infected with virus plus KKT.

**Fig. 4.** KKT affects viral protein synthesis. MDCK cells were either mock-infected or virus-infected at an m.o.i. of 0.01 in the presence of 4 μg/μl KKT or medium only. Cell lysate prepared at the indicated time point were subjected to Western blotting using antibody against NP, M1 and GAPDH.

**Fig. 5.** KKT did not inhibit viral RNP activity. (A) RNP activity assay by primer
extension assay in the presence of KKT. Different concentrations of KKT were incubated with HEK293 cells previously transfected with pHW2000-PA, PB1, PB2, NP, and pPOLI-CAT-RT. Total RNA was extracted and mixed with specific 32P-labeled primers and extended with AMV reverse transcriptase. The reaction products were denatured at 90 °C for 10 min, separated on 8% polyacrylamide gels containing 7 M urea and detected by autoradiography. (B) Luciferase reporter assay in the presence of various concentrations of KKT. HEK293 cells were transfected with pHW2000-PA, PB1, PB2, and NP plus pPOLI-Fluc reporter and pRL-TK as a transfection control. KKT was added 8 h post transfection and incubated for further 24 h. Treatment with KKT did not reduce the ratio of Fluc (firefly luciferase) to Rluc (renilla luciferase) activity, indicating that KKT did not inhibit RNP complex mediated transcription of pPOLI-Fluc. 3P is an RNP inhibitor (J-T Horng, unpublished data).

**Fig. 6.** KKT inhibits nuclear export of viral RNP complex at 9 h after viral infection. (a) MDCK cells were infected with the WSN influenza virus (moi = 1) in the absence, or presence of KKT (4μg/μl) (b).
Table 1. KKT inhibition spectrum against recent clinical influenza viruses and oseltamivir resistant viruses

<table>
<thead>
<tr>
<th>Cytotoxic effect</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>MDCK</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Influenza virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/WSN/33 (H1N1)</td>
<td>1.84 ± 0.63</td>
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<tr>
<td>A/3446/02/TW (H3N2)</td>
<td>1.06 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>A/5779/98/TW (H1N1)</td>
<td>0.98 ± 0.23</td>
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</tr>
<tr>
<td>A/058/09/TW (H1N1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.68 ± 0.85</td>
<td></td>
</tr>
<tr>
<td>A/066/09/TW (H1N1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.34 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>A/147/09/TW (H1N1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt; 5</td>
<td></td>
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<tr>
<td>A/70299/(H3N2)</td>
<td>0.95 ± 0.36</td>
<td></td>
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<tr>
<td>B/99/07/TW</td>
<td>&gt; 5</td>
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</tr>
<tr>
<td>B/710/05/TW</td>
<td>&gt; 5</td>
<td></td>
</tr>
<tr>
<td>B/70233/05/TW</td>
<td>&gt; 5</td>
<td></td>
</tr>
<tr>
<td>B/70325/05/TW</td>
<td>&gt; 5</td>
<td></td>
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</tbody>
</table>

<sup>a</sup>CC<sub>50</sub> was determined with an MTT assay

<sup>b</sup>IC<sub>50</sub> was determined by neutralization assay

<sup>c</sup>clinical oseltamivir resistant strains (Shih et al 2010)
Table 2

<table>
<thead>
<tr>
<th>Chinese herbs</th>
<th>Concentration (μg/μl)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>CC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>SI&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pueraria root (葛根, <em>Puerariae Radix</em>)</td>
<td>&gt; 7.98</td>
<td>7.98 ± 0.71</td>
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<tr>
<td>Peony root (白芍, <em>Paeoniae Lactiflorae Radix</em>)</td>
<td>&gt; 6.52</td>
<td>6.52 ± 0.79</td>
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<tr>
<td>Ma huang (Ephedrae Herba) (大黄)</td>
<td>0.44 ± 0.15</td>
<td>1.7 ± 0.83</td>
<td>3.8</td>
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<tr>
<td>Cinnamon twigs (Cinnamomi Ramulus) (肉桂)</td>
<td>1.53 ± 0.41</td>
<td>3.52 ± 0.57</td>
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<tr>
<td>Ginger (Zingiberis Siccatum Rhizoma) (姜)</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td></td>
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<tr>
<td>Jujube date (大枣, <em>Zizyphi Sativae Fructus</em>) (紅棗)</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td></td>
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<tr>
<td>Licorice (甘草, <em>Glycyrrhizae Radix</em>)</td>
<td>&gt; 1.57</td>
<td>1.57 ± 0.24</td>
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</tr>
</tbody>
</table>

<sup>a</sup>SI (selectivity index) is the ratio of CC<sub>50</sub> to IC<sub>50</sub>
Fig. 1.

Virus adsorption

-3 ~ -1
-1 ~ 0
0 ~ 1
1 ~ 10

Virus titer (PFU ml⁻¹)

-3-1
-1-0
0-1
1-10

Virus KKT
Fig. 2.

A

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>3h</th>
<th>6h</th>
<th></th>
<th>virus</th>
<th>KKT</th>
<th>Ly294002</th>
<th>DMSO</th>
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<tr>
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<td>+</td>
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P-Akt

NP

Total Akt

B

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<tr>
<th></th>
<th>5h</th>
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<td>-</td>
<td>KKT</td>
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<td>-</td>
<td>Ly294002</td>
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</tbody>
</table>

3h

KKT

LY294002

5% serum

P-Akt

Total Akt

GAPDH

1  2  3  4
Fig. 3A

![Graph showing virus titer (PFU ml⁻¹) over time for WSN (H1N1) virus. The graph includes the virus control line and Ko Ken Tang's line.](image-url)
Figure 3B

<table>
<thead>
<tr>
<th></th>
<th>Mock</th>
<th>8h</th>
<th>16h</th>
<th>24h</th>
<th>KKT</th>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- NP
- M1
- GAPDH
Figure 3C

a. Control  b. WSN

c. KKT  d. WSN+KKT
Figure 4

RNP Activity Inhibition Assay

*KKT concentration (μg/μl)*

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>M</td>
<td></td>
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</tbody>
</table>

Luciferase level

- vRNA
- mRNA
- cRNA

KKT concentration (μg/μl)
Figure. 5. KKT inhibits nuclear export of viral RNP complex at 9 h after viral infection. (a) MDCK cells were infected with the WSN influenza virus (moi = 1) in the absence, or presence of KKT (4μg/μl) (b).