Mycophenolic acid, an immunomodulator, has potent and broad-spectrum \textit{in vitro} antiviral activity against pandemic, seasonal and avian influenza viruses affecting humans

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Immunomodulators have been shown to improve the outcome of severe pneumonia. We have previously shown that mycophenolic acid (MPA), an immunomodulator, has antiviral activity against influenza A/WSN/1933(H1N1) using a high-throughput chemical screening assay. This study further investigated the antiviral activity and mechanism of action of MPA against contemporary clinical isolates of influenza A and B viruses. The 50 % cellular cytotoxicity (CC\textsubscript{50}) of MPA in Madin Darby canine kidney cell line was over 50 \textmu M. MPA prevented influenza virus-induced cell death in the cell-protection assay, with significantly lower IC\textsubscript{50} for influenza B virus B/411 than that of influenza A(H1N1)pdm09 virus H1/415 (0.208 vs 1.510 \textmu M, $P=0.0001$). For H1/415, MPA interfered with the early stage of viral replication before protein synthesis. For B/411, MPA may also act at a later stage since MPA was active against B/411 even when added 12 h post-infection. Virus-yield reduction assay showed that the replication of B/411 was completely inhibited by MPA at concentrations $>0.78$ \textmu M, while there was a dose-dependent reduction of viral titer for H1/415. The antiviral effect of MPA was completely reverted by guanosine supplementation. Plaque reduction assay showed that MPA had antiviral activity against eight different clinical isolates of A(H1N1), A(H3N2), A(H7N9) and influenza B viruses (IC\textsubscript{50} < 1 \textmu M). In summary, MPA has broad-spectrum antiviral activity against human and avian-origin influenza viruses, in addition to its immunomodulatory activity. Together with a high chemotherapeutic index, the use of MPA as an antiviral agent should be further investigated \textit{in vivo}.

\textbf{INTRODUCTION}

Both influenza A and influenza B viruses commonly cause respiratory tract infections in humans. Despite advances in critical care support, antivirals and vaccines, influenza virus infection is still associated with significant mortality, especially those caused by the avian-origin influenza A(H5N1)
Neuraminidase inhibitors are currently the mainstay of antiviral therapy. However, many patients still succumbed despite the early use of neuraminidase inhibitors (Chen et al., 2013; To et al., 2014a; Yu et al., 2013). The effectiveness of neuraminidase inhibitors has been evaluated in several meta-analyses, but authors differed in their conclusions (Dobson et al., 2015; Heneghan et al., 2014; Jefferson et al., 2014; Muthuri et al., 2014). Furthermore, there is an increasing number of viral isolates resistant to neuraminidase inhibitors, and resistance can emerge during treatment (Cheng et al., 2012; Takashita et al., 2015; To et al., 2013). Adamantanes are active against influenza A virus only, but are now rarely used because of resistance (Cheng et al., 2012). Favipiravir, a novel viral RNA polymerase inhibitor with broad-spectrum antiviral activity including anti-influenza activity, has been approved in Japan, but is still undergoing phase 3 clinical trials in the United States (Cao et al., 2014; ClinicalTrials.gov identifier: NCT02008344; Daikoku et al., 2014; Smee et al., 2013). Arbidol, which inhibits several steps of the influenza virus life cycle, has been approved for clinical use in Russia and China (Blaising et al., 2014). A recent randomized controlled trial in Russia showed that this drug can hasten the resolution of symptoms (Kiselev et al., 2015). Other novel antivirals, such as DAS181, are still undergoing clinical trials (Zumla et al., 2014). Convalescent blood products, which have both antiviral and immunomodulatory activity, have improved the survival of patients in the 1918 and 2009 A(H1N1) pandemics (Hung et al., 2011, 2013; Luke et al., 2006). However, these convalescent blood products are not widely available.

Although new compounds with high in vitro antiviral activity and in vivo efficacy have been found (Watanabe & Kawaoka, 2015), clinical use of these novel agents requires further modification of the drug to optimize pharmacokinetics and pharmacodynamics, and a lengthy review process in humans to establish the safety profile. The pharmacokinetics, pharmacodynamics and adverse effects of drugs that are already approved for human use are well described, and therefore repurposing of these drugs for treatment of severe influenza will dramatically reduce the time for regulatory processes. Nitazoxanide, an approved antiparasitic agent with broad-spectrum antiviral activity, could reduce the duration of symptoms in patients with uncomplicated influenza virus infection, but the efficacy in patients with severe illnesses has not been reported (Haffizulla et al., 2014). The combination of celecoxib with neuraminidase inhibitors has shown to exert a synergistic effect in a mouse model (Zheng et al., 2008); and naproxen decreases pulmonary titers in infected mice (Lejal et al., 2013), but no human studies have been reported for these clinically approved non-steroidal anti-inflammatory agents.

We have previously used a high-throughput chemical screening platform to search for novel antivirals and discovered that nucleozin has potent antiviral activity and was able to protect mice from lethal infection (Kao et al., 2010). Using the same platform, we have also screened 1280 compounds for anti-influenza activity. Mycophenolic acid (MPA) was found to have a 50 % inhibitory concentration (IC$_{50}$) of 0.24 µM, a 50 % toxic concentration (TC$_{50}$) of >100 µM and a selectivity index of >708 against influenza A(H1N1) virus A/WSN/1933 (Chan et al., 2013a). MPA is a non-competitive reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH). MPA, or its prodrug mycophenolate mofetil, has been widely used as an immunomodulator in patients with autoimmune diseases and transplant recipients (Villarroel et al., 2009). In this study, we have evaluated the antiviral activity of MPA against the contemporary clinical isolates, including representative strains from the pandemic A(H1N1)pdm09 virus, seasonal A(H3N2) virus, and avian-origin influenza A(H7N9) viruses. We have also determined its mechanism of action.

## RESULTS

### Cytotoxicity of MPA

To confirm the cytotoxicity results from our previous high-throughput chemical library screening (Kao et al., 2010), we have performed the cytotoxicity assay in Madin Darby canine kidney (MDCK) cells using the methylthiazolylidiphenyl-tetrazolium bromide (MTT) assay. The 50 % cellular cytotoxicity (CC$_{50}$) of MPA was over 50 µM (Fig. 1).

![Fig. 1: Cytotoxicity of mycophenolic acid on Madin Darby canine kidney (MDCK) cells. Cell survival was measured using methylthia-zolyldiphenyl-tetrazolium bromide assay. Data represents the mean of two experiments with triplicate in each experiment. Error bars indicate standard error of mean.](chart)

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MPA inhibited both influenza A and B viruses using cell-viability assay

First, we sought to confirm the antiviral activity of MPA against influenza viruses by cell-viability assay that was shown in our previous robotic screening (Chan et al., 2013a). Clinical strains of influenza viruses used in this study are listed in Table 1. MDCK cells were infected by virus at an MOI of 0.0001, and MPA was added 2 h before virus infection. Cell viability was determined using MTT assay on day 3 post-infection. As a control, we evaluated the antiviral activity in parallel with zanamivir. The 50 % inhibitory concentration (IC\textsubscript{50}) of MPA was lower for B/411 (0.208 µM) than that of the 2009 pandemic H1/415 (1.510 µM) (\(P=0.0001\)) (Table 2). The IC\textsubscript{50} of MPA for H1/415 was similar to those of zanamivir, but the IC\textsubscript{50} for B/411 was much lower for MPA (0.208 µM) than zanamivir (>12.5 µM). Therefore, MPA has antiviral activity against both influenza A and B viruses at low concentrations.

MPA affects the early stage of viral replication for influenza A virus, but may also affect the late stage for influenza B virus

To determine the stage of the viral life cycle affected by MPA, time-of-addition assay was performed. Cell viability was determined using MTT assay at pre-determined time points post-infection. For H1/415, there was no significant difference in the cell viability when MPA was added at 2 h before infection (\(T=-2\)), at the time of virus inoculation (\(T=0\)) or 1 h post-infection (\(T=1\)), but the cell viability was significantly lower when MPA was added 7 h post-infection (\(T=7\)) (\(P<0.0001\)) (12.5 µM) and \(P<0.001\) (3.125 µM) when comparing between \(T=7\) and \(T=-2\) (Fig. 2). In contrast, the neuraminidase inhibitor zanamivir, which acts at the last stage of the viral life cycle during viral release, exhibited significant cell protection even when added 7 h post-infection (Fig. 2b).

For B/411, there was no significant difference when MPA was added between 2 h before virus inoculation and 7 h post-infection. Therefore, we further delayed the time of addition of MPA. While there was still significant cell protection at 12 h post-infection (\(T=12\)), the protection was much reduced at 24 h post-infection (\(T=24\)) (\(P<0.0001\) when comparing between \(T=-2\) and \(T=24\) when MPA concentrations were at 12.5 or 3.125 µM) (Fig. 2c). On the other hand, zanamivir exhibited much poorer cell protection for B/411 than that of H1/415 (Fig. 2d). The results suggest that MPA acted at an early stage of the virus life cycle for influenza A virus, while it may also act at a later stage for influenza B virus.

MPA inhibited viral replication

The effect of MPA on viral replication was assessed using the virus yield reduction assay (Fig. 3). The growth of B/411 was completely abolished by MPA at a concentration of 0.78 µM or above, while live virus was still detected with zanamivir at a concentration of 12.5 µM. For H1/415 and H7/AH1, there was a dose-dependent reduction of viral titers by MPA although

<p>| Table 1. Clinical strains of influenza viruses used in this study |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Influenza virus subtype</th>
<th>Year of isolation</th>
<th>Virus strain</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(H1N1)</td>
<td>2009</td>
<td>A/Hong Kong/415742/2009</td>
<td>H1/415</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>A/Hong Kong/402467/2014</td>
<td>H1/402</td>
</tr>
<tr>
<td>A(H3N2)</td>
<td>2011</td>
<td>A/Hong Kong/447572/2011</td>
<td>H3/447</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>A/Hong Kong/460611/2013</td>
<td>H3/460</td>
</tr>
<tr>
<td>A(H7N9)</td>
<td>2013</td>
<td>A/Anhui/1/2013</td>
<td>H7/AH1</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>A/Zhejiang/DTID-ZJU01/2013</td>
<td>H7/ZJ1</td>
</tr>
<tr>
<td>B</td>
<td>2011</td>
<td>B/Hong Kong/411989/2011</td>
<td>B/411</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>B/Hong Kong/446321/2013</td>
<td>B/446</td>
</tr>
</tbody>
</table>

| Table 2. IC\textsubscript{50} of MPA and zanamivir against influenza viruses in cell protection assay |
|---------------------------------|-----------------|-----------------|-----------------|
| Influenza virus subtype | Virus strain | IC\textsubscript{50} (µM) (95% C.I.)* |
|--------------------------|-----------------|-----------------|-----------------|
|                          | MPA             | Zanamivir       |
| A(H1N1)          | H1/415          | 1.51 (0.801–2.846) | 0.864 (0.202–3.686) |
| B                | B/411           | 0.208 (0.109–0.393) | >12.5 |

C.I., confidence interval; IC\textsubscript{50}, 50 % inhibitory concentration; MPA, mycophenolic acid.

*Cell viability was determined using the methylthiazolyldiphenyl-tetrazolium bromide assay. IC\textsubscript{50} was determined using the three-parameter logistic nonlinear regression model equation in GraphPad Prism 6 software. The IC\textsubscript{50} was determined using the results from two independent experiments, with triplicates in each experiment.
the inhibitory effect was less than that of B/411. Therefore, MPA could inhibit viral replication.

MPA inhibits protein expression of influenza A and B viruses
To determine whether MPA acts before or after the protein synthesis step in the viral life cycle, viral protein expression was assessed at 6 h post-infection by immunofluorescence assay. MDCK cells were infected at a high MOI of 1, and MPA was added 2 h prior to infection. MPA at a concentration of 12.5 µM and 3.125 µM inhibited protein expression by >95 % for both H1/415 and B/411 virus (Fig. 4), which suggests that MPA inhibited protein synthesis or replication steps before protein synthesis.

While the inhibition of H1/415 was significantly reduced when the MPA concentration was at 0.78 µM and almost no inhibition at 0.2 µM, the inhibition of B/411 by MPA was still >50 % at a concentration of 0.2 µM. Since this difference in the inhibition of protein expression could be related to the timing of protein expression, we performed Western blot analysis from 0 to 6 h post-infection. Nucleoprotein was expressed at similar time interval after infection for H1/415 and B/411 (Fig. S1, available in the online Supplementary Material). Therefore, the lower concentration of MPA
Guanosine supplementation reverted the antiviral activity of MPA

Since MPA depletes the intracellular guanosine pool by inhibiting IMPDH (Allison & Eugui, 2000; Olschlager et al., 2011), we investigated whether the addition of guanosine would revert the antiviral effect of MPA. The addition of guanosine, but not adenosine, reverted the antiviral activity of MPA for both H1/415 and B/411 (Fig. 5a). On the other hand, there was reversal of anti-H1N1 activity for ribavirin when either adenosine or guanosine was added (Fig. 5b). Therefore, guanosine depletion has contributed to the antiviral activity of MPA.

MPA has broad-spectrum antiviral activity against both influenza A and B viruses

Using plaque reduction assay, we determined the IC_{50} of MPA against different clinical isolates of A(H1N1), A (H3N2), A(H7N9) and influenza B viruses (Table 3). The IC_{50} of MPA was found to be similar for different strains of seasonal or pandemic influenza A and B viruses (range, 0.344–0.630 µM). The IC_{50} of MPA against A(H7N9) viruses were slightly higher (0.872 and 0.919 µM for H7/AH1 and H7/ZJ1, respectively). Therefore, MPA has broad antiviral activity against influenza viruses.

DISCUSSION

Currently available antivirals against influenza A and B viruses have limited clinical efficacy (Jefferson et al., 2014; Muthuri et al., 2014). In our previous high-throughput chemical screening assay, MPA exhibited antiviral activity against the historical A(H1N1) strain A/WSN/1933 (Chan et al., 2013a). In the current study, we have confirmed that MPA also has broad-spectrum antiviral activity against contemporary clinical strains of influenza A and B viruses. Using time-of-addition assay and protein-expression assay, we deduce that MPA acts at an early stage of infection before protein synthesis. Guanosine supplementation abolished the antiviral activity of MPA. Taken together, these results suggest that the inhibition of influenza virus replication by MPA is attributable to host-cell guanosine depletion, leading to inhibition of either genome replication or protein synthesis. This is consistent with the known effects of MPA on cell replication. MPA is a non-competitive reversible inhibitor of IMPDH, which is an enzyme involved in the cellular biosynthesis of purine mononucleotides (Villarroel et al., 2009). MPA has broad-spectrum...
antiviral activity against both DNA and RNA viruses (Chan et al., 2013a; Chapuis et al., 2000; Cline et al., 1969; Diamond et al., 2002; Khan et al., 2011; Sebastian et al., 2011; Wang et al., 2014b; Ye et al., 2012), and guanosine depletion has been found to contribute to antiviral activity for several of these viruses.

There are several favourable attributes which suggest that MPA may be used in the treatment of influenza virus infections in humans before the availability of more effective drugs. Firstly, mycophenolate mofetil (MMF), the prodrug of MPA, and the enteric-coated form, mycophenolate sodium, have been approved for clinical use for many years, and therefore the safety profile, pharmacokinetics and pharmacodynamics are well known (van Gelder & Hesselink, 2015). Repurposing of MMF or mycophenolate sodium for treatment of influenza will avoid the long regulatory process associated with the approval of a novel compound. Secondly, since the $C_{\text{max}}$ of MPA in humans [25 µg ml$^{-1}$ (78 µM) after 1000 mg single oral dose of MMF, 26 µg ml$^{-1}$ (81 µM) after 720 mg single oral dose of mycophenolate sodium] is much higher (>50 times) than the IC$_{50}$ demonstrated in our in vitro assays, the concentration of MPA in humans should be sufficient to inhibit viral replication (Anonymous). In fact, the IC$_{50}$ of MPA is comparable with that of nitazoxanide (0.9–3.2 µM) and favipiravir (0.19–22.5 µM), which have undergone human clinical trials in the treatment of influenza virus infection (Belardo et al., 2015; Furuta et al., 2013). Thirdly, since this drug targets the viral replication by depleting the host guanosine pool, it is unlikely that the virus can develop resistance against it. Many studies are now looking at similar drugs targeting host factors (Watanabe et al., 2014). Fourthly, MPA is an immunomodulator which can dampen the excessive inflammatory response associated with severe influenza. It was shown that cytokine dysregulation is evident in severe influenza virus infection (Chen et al., 2013; To et al., 2010), and the use of convalescent plasma and hyperimmune globulin have been shown to improve survival (Hung et al., 2011, 2013). In a mouse model, MMF has been shown to reduce the level of cytokines in acute lung injury due to lipopolysaccharide (LPS) (Beduschi et al., 2013). Another mouse model showed that MMF improved the survival of mice with LPS-induced liver failure (Yang et al., 2008). MMF has also been shown to improve lung function in patients with interstitial lung disease (Fischer et al., 2013).

There are concerns about the use of immunomodulators during acute infection. However, several immunomodulators have been shown as beneficial during acute pneumonia. A short course of corticosteroid has been shown to hasten clinical improvement or reduce treatment failure in double-blind randomized placebo-controlled trials (Blum et al., 2015; Torres et al., 2015). The rapamycin inhibitor sirolimus, an immunomodulator frequently used in transplant recipients, was shown to improve the outcome of patients in addition to corticosteroid in a randomized controlled trial involving patients with severe A(H1N1) pneumonia.

Fig. 4. Inhibition of influenza A and influenza B virus protein expression by mycophenolic acid (MPA) at 6 h post-infection. Protein expression was determined using fluorescein-tagged murine monoclonal antibodies against influenza A or influenza B virus proteins. Percentage of inhibition by MPA was calculated by comparing infected cells with or without the addition of MPA. The experiment was performed in triplicate. Error bars indicate standard error of mean.

Fig. 5. The effect of the addition of guanosine or adenosine on the antiviral effect of mycophenolic acid (MPA) (a) and ribavirin (b) in plaque reduction assays. Each experiment was performed in duplicate, and the results were the means of the two independent experiments.
(Wang et al., 2014a). Since the $C_{\text{max}}$ achieved by MMF or mycophenolate sodium is much higher than the $IC_{50}$ against influenza viruses, a much lower dose of these drugs with minimal immunomodulatory activity should be sufficient for antiviral activity in humans. It has been found that the effect on CD4+ T cells is much less for low-dose MMF (100 mg or 250 mg) than that of usual dose of MMF (1000 mg) (Vethe et al., 2008). Nevertheless, further animal and human studies should be conducted to ascertain the safety of using these immunomodulators including mycophenolate as their effect on suppression of the host immune system, although expected to be moderate, may surpass their beneficial antiviral effect.

Neuraminidase inhibitor is the only class of antiviral approved for the treatment of influenza B virus infection. In vitro, the $IC_{50}$ of zanamivir is generally higher than that of influenza A virus (Sheu et al., 2008). Recent influenza B virus isolates showed reduced susceptibility to neuraminidase inhibitors due to the emergence of N294S and I221V mutation of the neuraminidase (Carr et al., 2011; Garg et al., 2013), and high-level resistance, due to I221L mutation, has been reported (Escuret et al., 2014). Clinical studies have shown that neuraminidase inhibitors are less effective against influenza A viruses (Burnham et al., 2013). Oseltamivir was not effective in the prophylaxis of influenza B virus infection (Burnham et al., 2013). By our cell-protection assay, we have confirmed that zanamivir has poorer antiviral activity against influenza B than A viruses. On the other hand, MPA has shown good activity against influenza A virus. In fact, the antiviral activity of MPA against influenza B virus is more potent than that of influenza A virus in the virus yield reduction assay, cell-protection assay and protein-expression assay. Notably, in virus yield reduction assay, MPA could completely inhibit the growth of influenza B virus. There are several differences that may account for the difference in antiviral activity of MPA against influenza A and B viruses. Firstly, influenza B virus has a slower replication rate than influenza A virus (Fig. 3), and may therefore be more susceptible to the effect of guanosine depletion. Secondly, there are differences in their polymerase proteins. The polymerase activity has been reported to be reduced when PB1 of influenza A virus is replaced by PB1 of influenza B virus. The PA protein of influenza B virus has also been found to have no affinity to PB1 of influenza A virus (Wunderlich et al., 2010). Besides, in the synthesis process of viral mRNA, viral polymerase of influenza B virus has been reported to be less dependent on the methyl residue than that of influenza A virus in recognizing the cap structure (Wakai et al., 2011). It is because, unlike influenza A virus, the cap-recognition pocket of PB2 protein of influenza B virus is not specific to methylated residue of cap. Furthermore, the PB1 of influenza B virus, but not PB1 of influenza A virus, contains global ordered RNA structure (GORS), which is associated with the secondary structure of RNA (Priore et al., 2012, 2013) The secondary structure of RNA may be associated with the stability. Since intertypic reassortants consisting of engineered genes containing open-reading frames of influenza A and B virus proteins can be generated, such reassortants may provide further insights into the difference in antiviral activity of MPA (Baker et al., 2014).

There are several limitations in our study. Firstly, we cannot exclude alternative possible antiviral mechanisms other than depletion of guanosine pool. MPA may also target other pathways of viral replication. For influenza B virus, significant inhibition was noted, even when MPA was added 12 h post-infection, suggesting that MPA may interfere with a step after genome replication/protein synthesis. Secondly, we have tested a limited number of strains for each virus subtype. More strains should be tested to assess whether it is a general phenomenon that MPA possesses a more potent antiviral activity for influenza B than that of influenza A viruses. Thirdly, we have investigated the antiviral activity of MPA on influenza virus in MDCK cell line only. The antiviral activity of a drug may vary depending on the cell lines used in the experiment. For example, ribavirin is not

### Table 3. $IC_{50}$ of MPA and zanamivir against influenza viruses in plaque reduction assay

<table>
<thead>
<tr>
<th>Influenza virus subtype</th>
<th>Virus strain</th>
<th>MPA (μM) (95% C.I.)*</th>
<th>Zanamivir (μM) (95% C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(H1N1)</td>
<td>H1/415</td>
<td>0.624 (0.368–1.060)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>H1/402</td>
<td>0.344 (0.171–0.694)</td>
<td>0.182 (0.101–0.328)</td>
</tr>
<tr>
<td>A(H3N2)</td>
<td>H3/447</td>
<td>0.348 (0.186–0.653)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>H3/460</td>
<td>0.558 (0.268–1.161)</td>
<td>0.294 (0.125–0.691)</td>
</tr>
<tr>
<td>B</td>
<td>B/411</td>
<td>0.528 (0.257–1.088)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B/446</td>
<td>0.630 (0.408–0.971)</td>
<td>0.030 (0.013–0.071)</td>
</tr>
<tr>
<td>A(H7N9)</td>
<td>H7/AH1</td>
<td>0.872 (0.473–1.609)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>H7/ZJ1</td>
<td>0.919 (0.605–1.395)</td>
<td>0.131 (0.076–0.224)</td>
</tr>
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</table>

C.I., confidence interval; $IC_{50}$, 50 % inhibitory concentration; MPA, mycophenolic acid.

* $IC_{50}$ was determined using the three-parameter logistic nonlinear regression model equation in GraphPad Prism 6 software. The experiment was performed in triplicate.
active against severe acute respiratory syndrome coronavirus in Vero cells, but active in five other cell lines (Morgenstern et al., 2005). The nucleotide sequences of the IMPDH2 of Homo sapiens (NC_012091) and that of Canis lupus familiaris (NC_006602) have a similarity of only 71.4%. Therefore, the IMPDH2 in the MDCK cells is likely to differ from that of human cells. The effect of MPA on influenza viruses should be further investigated in other cell lines of different animal- and different human-tissue origin. In summary, this study demonstrated substantial antiviral activity of MPA against diverse subtypes of influenza viruses. Further in vivo studies are necessary to determine the efficacy of MPA in animals and in humans.

METHODS

Viruses, cells, and compounds. All virus strains used in this study were isolated from patients (Table 1). The virus strains H1/415, H7/ AH1 and H7/Z21 were used in our previous studies (Chan et al., 2013b; Chen et al., 2013; Li et al., 2009; To et al., 2013; Zheng et al., 2008). These viruses were propagated in MDCK cells before use. MPA (Sigma, St. Louis, Missouri), prepared as 100 mM stocks in 100% dimethyl sulfoxide (DMSO) and zanamivir (GlaxoSmithKline, Boronia, Australia), prepared as 25 mM stocks in water and were stored at −20°C until use. Working solutions of MPA and zanamivir were prepared on the day of experiment.

Cytotoxicity assay. The cytotoxicity of MPA and zanamivir in MDCK cells was determined using MTT assay according to the manufacturer’s instruction as we described previously (Sigma, St. Louis, Missouri) (Chan et al., 2013a).

Time of addition experiment using cell viability assay. Influenza viruses at a multiplicity of infection (MOI) of 0.0001 were inoculated onto MDCK cells in minimum essential medium (MEM) (Gibco, N.Y., USA) in 12-well plates for 2 h. Then, influenza viruses were added at 160 MOI of 1 and MPA at predetermined concentrations were added to the cells at 2 h before virus inoculation. For the control group, DMSO alone was used instead of MPA. At predetermined time points post-infection, the supernatants were collected for virus quantification using plaque assay as described previously (Kao et al., 2010). Each experiment was performed in duplicate, and the results were the means of two independent experiments.

Virus yield reduction assay. Influenza viruses at an MOI of 0.0002 were inoculated onto MDCK cells in MEM with 2 µg ml⁻¹ of TPCK-treated trypsin and incubated at 37°C for 1 h. Non-attached viruses were removed by washing the cells twice in serum-free MEM. The monolayer cells were maintained in MEM with 2 µg ml⁻¹ of TPCK-treated trypsin. MPA or zanamivir in fourfold dilutions were added to the cells at 2 h before virus inoculation. For the control group, DMSO alone was used instead of MPA. At predetermined time points post-infection, the supernatants were collected for virus quantification using plaque assay as described previously (Kao et al., 2010). Each experiment was performed in duplicate, and the results were the means of two independent experiments.

Immunofluorescence assay for viral protein expression. The effect of MPA on influenza A and B viral protein expression was assessed by immunofluorescence assay as described previously with modification (Chan et al., 2011; Khan et al., 2011). MDCK cells were treated with MPA at 37°C for 2 h. After washing with serum-free MEM, virus at an MOI of 1 and MPA at predetermined concentrations were added to the cells and incubated at 37°C for 1 h. After washing, MPA was replenished. At 6 h post-infection, the seeded cells were fixed in chilled acetone at −20°C for 10 min and stained with fluorescein-tagged mouse monoclonal antibodies against influenza A and B virus proteins (influenza A and influenza B DFA Reagent, D® Ultra 8™ DFA Respiratory Virus Screening and Identification Kit, Diagnostic Hybrids, Inc. Quidel, USA) at 37°C for 30 min and examined under fluorescence microscope. The percentage of positive cells was determined. Each experiment was performed in triplicate.

Western blotting. Western blotting analysis of nucleoprotein expression was performed as described previously with modification (Mok et al., 2012). Cell lysates were fractionated by 10% SDS-PAGE and then blotted onto polyvinyl difluoride (PVDF) membranes. Membranes were incubated with primary antibodies including mouse anti-beta-actin antibody (diluted 1:7000; Sigma), goat anti-influenza A virus antibody (diluted 1:1000; AB1074; Merck Millipore) or mouse anti-influenza B antibody (diluted 1:500) (Qiu et al., 2009). Membranes were then incubated with horseradish peroxidase-labeled rabbit anti-goat antibody (1:4000; Invitrogen) or goat anti-mouse IgG secondary antibody (1:3000; BioRad). Blots were visualized using ECL Plus Western blotting detection reagents (Avansta).

Statistical analysis. The 50% cellular cytotoxicity (CC₅₀) and 50% inhibitory concentration (IC₅₀) were determined using the three-parameter logistic nonlinear regression model equation in GraphPad Prism 6 software as described previously (Baranovich et al., 2013). For the comparison of the cell protection between different time points for the time-of-addition assay, two-way ANOVA with Tukey correction for multiple comparisons was used.

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