Combination of oseltamivir and fibrates prolong the mean survival time of mice infected with the lethal H7N9 influenza virus

Lili Xu,1 Linlin Bao,1 Fengdi Li,1 Songzhi Gu,1 Qi Lv,1 Jing Yuan,1 Yanfeng Xu,1 Hua Zhu,1 Wei Deng,1 Yanhong Li,1 Yanfeng Yao,1 Pin Yu,1 Zhancheng Gao2 and Chuan Qin1

1Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (CAMS) and Comparative Medicine Center, Peking Union Medical College (PUMC), Key Laboratory of Human Disease Comparative Medicine, Ministry of Health, Beijing, PR China

2Department of Respiratory and Critical Care Medicine, Peking University People’s Hospital, Beijing, PR China

The outbreak of human infections caused by the novel avian-origin H7N9 influenza viruses in China since March 2013 underscores the urgent need to find an effective treatment strategy against H7N9 infection in humans. In this study, we assessed the effectiveness of combinations of oseltamivir and two immunomodulators (simvastatin and fenofibrate) against H7N9 infection in a mouse model. Mice treated with oseltamivir plus fenofibrate exhibited the longest mean survival time, the largest reduction of viral titre in lung tissue, the highest levels of CD4+ and CD8+ T-lymphocytes, and the greatest decrease in pulmonary inflammation. Thus, the combination of oseltamivir plus fenofibrate improved the outcomes of mice infected with H7N9 virus by simultaneously reducing viral replication and normalizing the aberrant immune response. This drug combination should be considered in randomized controlled trials of treatments for H7N9 patients.

In March 2013, a novel avian-origin H7N9 subtype influenza virus was identified as the causative agent of influenza-like illnesses in humans in eastern China (Gao et al., 2013). Most patients presented with respiratory infections that progressed to severe pneumonia and dyspnoea (Gao et al., 2013). Although the virus did not spread efficiently amongst humans, limited, non-sustained human-to-human transmission could not be excluded in a few family clusters (Li et al., 2014).

There is an urgent need to find an effective treatment strategy for H7N9 infection in humans. Although vaccines are the most effective means of controlling influenza infections, none are available for the prevention of H7N9 infections (Osterholm et al., 2013). Moreover, H7N9 viruses are resistant to adamantanes because they harbour the S31N amino acid substitution in the M2 protein; thus, neuraminidase (NA) inhibitors are the only option for the control of H7N9 influenza infections (Gao et al., 2013). Oseltamivir is recommended for the treatment of human H7N9 infections (Li et al., 2014). It has been reported that oseltamivir at 20 and 80 mg kg⁻¹ protected 80 and 88% of mice, respectively, when treatment was initiated 24 h after the mice were inoculated with 3 LD₅₀ of the A/Anhui/1/2013 (H7N9) virus. Moreover, the efficacy decreased to 70 and 60%, respectively, when treatment was delayed until 48 h after inoculation (Baranovich et al., 2014). In addition, H7N9 viruses with oseltamivir resistance-associated mutations (R152K and R292K, N2 numbering) were reported, emphasizing the importance of monitoring the emergence of oseltamivir-resistant viruses (Gao et al., 2013; Hu et al., 2013) and exploring more effective treatments as soon as possible.

Our preliminary research revealed that triple combinations of oseltamivir with anti-inflammatory and immunomodulator statins and fibrates could improve the survival rate of mice inoculated with the lethal, highly pathogenic avian influenza H5N1 virus. Combinations of an inhibitor of the viral NA (oseltamivir) and two inhibitors of inflammation (simvastatin and fenofibrate) exhibited synergistic effects in the mice, significantly inhibiting body weight loss within 5 days of initiating treatment and delaying the mean time of death by 2 days (An et al., 2011). In the present study, we investigated whether combinations of oseltamivir, simvastatin and fenofibrate could also exhibit enhanced therapeutic efficacy in a mouse model infected with H7N9 (Xu et al., 2013).

Six groups of 10 female, 6-week-old, specific-pathogen-free BALB/c mice were inoculated intranasally with 10 LD₅₀ of the A/Anhui/1/2013 (H7N9) virus. Oseltamivir (Roche) and the immunomodulators simvastatin (Sigma-Aldrich)
and fenofibrate (Sigma) were administered by gavage once per day for 5 days, beginning 2 h post-inoculation (p.i.). The administered dosage of each agent is listed in Table 1. Statistical analysis of the differences in the mean survival time, virus titres, levels of cytokines and chemokines, and CD4+ and CD8+ T-lymphocytes were performed by SPSS 11.5 software, and the Duncan and least significant difference methods were used for detection in one-way ANOVA means. \( P < 0.05 \) was considered statistically significant.

None of the infected mice survived when treated with PBS or oseltamivir plus simvastatin. One mouse in the simvastatin plus fenofibrate treatment group (10%) survived. Amongst the mice treated with oseltamivir alone, oseltamivir plus fenofibrate or oseltamivir plus simvastatin and fenofibrate, two mice (20%) survived in each group (Fig. 1a). However, the mean survival time of the mice treated with oseltamivir plus fenofibrate was prolonged to 9.5 days, compared with 7.9 days in the mice treated with oseltamivir alone (Fig. 1b). Therefore, we selected the combination of oseltamivir and fenofibrate for further study.

An additional three groups of 25 BALB/c mice were then inoculated intranasally with 10 LD50 of the A/Anhui/1/2013 (H7N9) virus. Similar to the previous experiment, oseltamivir, oseltamivir plus fenofibrate or PBS was administered by gavage once per day for 5 days, beginning 2 h p.i. Five randomly selected mice were euthanized per day at 4, 6 and 8 days p.i. Blood, bronchoalveolar lavage fluid (BALF), lung tissues and brain tissues were collected for virological, immunological and histopathological assays (Table 1). Significant decreases (\( P < 0.01 \)) in the viral titre in lung tissues, measured by TCID50, were found in the group treated with oseltamivir plus fenofibrate at 4, 6 and 8 days p.i. There were also significant differences (\( P < 0.05 \)) between the viral titres of mice treated with oseltamivir alone and oseltamivir plus fenofibrate at 4, 6 and 8 days p.i. (Fig. 1c). The pathological analysis demonstrated that the alveolar damage and interstitial inflammatory infiltration exhibited by the mice treated with oseltamivir plus fenofibrate were much less severe than in those treated with oseltamivir alone. Amongst the mice treated with PBS, oseltamivir alone and oseltamivir plus fenofibrate, pathological lesions occurred in 90–100, 40–60 and 10–30% of specimens, respectively (Fig. 2c). However, no significant pathological changes or tissue damage were detected in brain tissues from any of the inoculated mice.

The levels of several inflammatory markers in serum and BALF samples of H7N9 infection (Mok et al., 2013; Zhou et al., 2013) were subsequently measured either by flow cytometry (FACSCanto; BD Biosciences) using a cytometric bead array kit or by ELISA (R&D Systems). Amongst these cytokines and chemokines, the levels of TNF-\( \alpha \) and IFN-\( \gamma \)-induced protein-10 (IP-10) were significantly decreased in both BALF and serum samples collected at 4, 6 and 8 days p.i. from mice treated with oseltamivir plus fenofibrate. The levels of macrophage inflammatory protein (MIP)-1\( \beta \) were also significantly lower in BALF obtained from the mice treated with oseltamivir plus fenofibrate (Fig. 2a). However, there were no significant differences between any of the inoculated mouse groups in the levels of MIP-1\( \alpha \), RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemoattractant protein (MCP)-1, MCP-3, keratinocyte-derived chemokine or IFN-\( \alpha \) in the BALF or serum samples.

Furthermore, blood cells from the mice were stained with fluorescently labelled mAbs specific for mouse CD3, CD4 and CD8 (BD Pharmingen), and the cells were fixed with 4% p-formaldehyde overnight. The proportions of different subsets of T-cells were determined by flow cytometry.

### Table 1. Treatment regimens containing oseltamivir, simvastatin and fenofibrate, either alone or in combination, in H7N9-inoculated mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Purpose</th>
<th>Treatment regimen (gavage route, once per day ×5 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 per group</td>
<td>Survival rate observation</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1}))</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1})) + simvastatin (50 mg kg(^{-1}))</td>
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<tr>
<td>4</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1})) + fenofibrate (150 mg kg(^{-1}))</td>
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<tr>
<td>5</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1})) + simvastatin (50 mg kg(^{-1})) + fenofibrate (150 mg kg(^{-1}))</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>Simvastatin (50 mg kg(^{-1})) + fenofibrate (150 mg kg(^{-1}))</td>
</tr>
<tr>
<td>7</td>
<td>25 per group (five mice per time point: 4, 6 and 8 days p.i.)</td>
<td>Virological, immunological and pathological analyses</td>
<td>PBS</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1}))</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>Oseltamivir (30 mg kg(^{-1})) + fenofibrate (150 mg kg(^{-1}))</td>
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</tbody>
</table>
Results showed that the levels of both CD4$^{+}$ and CD8$^{+}$ T-lymphocytes were significantly higher in the blood taken at 8 days p.i. or both 6 and 8 days p.i., respectively, from the mice given the combination therapy of oseltamivir plus fenofibrate than the blood samples taken from the mice treated with oseltamivir alone or PBS (Fig. 2b).

Mice are a well-established model for evaluating viral pathogenesis and the efficacy of antiviral drugs because mice and humans develop similar changes in the respiratory tract after viral infection, including the predominant involvement of the lower airway (Barnard, 2009). In this study, we assessed the effectiveness of combination treatments comprising oseltamivir plus an immunomodulator, either simvastatin or fenofibrate, against H7N9 infection in a mouse model. The mice treated with oseltamivir plus fenofibrate had prolonged mean survival times accompanied by significantly lower viral titres in lung tissue, higher levels of CD4$^{+}$ and CD8$^{+}$ T-lymphocytes, and decreased pulmonary inflammation.

It is now accepted that both excessive viral replication and overactive inflammatory responses contribute to poor prognoses in humans with influenza infections. It has been reported that combinations of a neuraminidase inhibitor (zanamivir) with two immunomodulators (celecoxib and mesalazine) could reduce mortality in mice infected by a high inoculum of H5N1 influenza virus (Zheng et al., 2008). Oseltamivir is a compound that mimics the natural substrate of NA and competes with the natural substrate for binding to the active site of NA. As a result, the progeny virions fail to be released from sialic acid receptors and aggregate on the surface of the infected cell, hampering the spread of infection to other, uninfected cells (Samson et al., 2013). Fibrates are agonists for peroxisome proliferator-activated receptor (PPAR)-$\alpha$, a ligand-activated transcription factor belonging to the nuclear receptor superfamily. PPAR-$\alpha$ has multiple biological effects, including the promotion of lipid biosynthesis and glucose metabolism, and it exhibits potent anti-inflammatory activity both in vitro and in vivo (Paola & Cuzzocrea, 2007). PPAR-$\alpha$ agonists can inhibit several intracellular signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway members p38-MAPK and Jun N-terminal kinases, which appear to be crucial for influenza virus replication (Ludwig et al., 2006). It has been reported that fibrates inhibit the cytokine-induced mRNA expression of several pro-inflammatory genes (Chinetti et al., 2000). In this study, we found that the activation of PPAR-$\alpha$ by fenofibrate significantly decreased the levels of IP-10, TNF-$\alpha$ and MIP-1$\beta$, all of which are key pro-inflammatory cytokines. Thus, the combination of oseltamivir plus fenofibrate may improve the outcomes of patients infected with H7N9 influenza virus by simultaneously suppressing viral replication and the inflammatory response.
Our study also showed that both CD4\(^+\) and CD8\(^+\) T-lymphocytes were significantly increased in the group treated with oseltamivir plus fenofibrate. However, unlike steroids or other immunosuppressants, treatment with oseltamivir plus fenofibrate allowed the maintenance of significantly higher levels of CD4\(^+\) and CD8\(^+\) T-lymphocytes at 6 and 8 days p.i.

However, Zheng et al. (2008) reported that zanamivir with or without immunomodulators reduced viral load to a similar extent. In this study, we found that fibrates affected the virus titres. It is known that both PPAR-\(\alpha\) and PPAR-\(\gamma\) agonists have anti-inflammatory and immunomodulatory activities, and several investigators have suggested that they might be used to treat acute lung injury (Becker et al., 2006; Cuzzocrea, 2006; Paola & Cuzzocrea, 2007). One study showed that glitazones (PPAR-\(\gamma\) agonists) inhibit respiratory syncytial virus infection in human lung epithelial cells, probably by inhibiting viral gene expression and not earlier adhesion or fusion processes (Arnold & König, 2006). No studies have reported the direct antiviral effects of PPAR-\(\alpha\) agonists, yet both PPAR-\(\alpha\) and PPAR-\(\gamma\) agonists affect several intracellular signalling pathways that are crucial for influenza virus replication (Gardner et al., 2005; Ludwig et al., 2006).

The ability of statins to inhibit cytokine production, improve endothelial cell function and modulate host molecular pathways has led to the hypothesis that statin use could be a preventative therapy against cellular damage caused by influenza virus infection (Blanc et al., 2011; Fedson, 2006), and our preliminary research showed that

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**Fig. 2.** Pro-inflammatory cytokines, T-lymphocyte counts in peripheral blood and pathological changes in lung tissues of H7N9-inoculated mice treated with various combinations of oseltamivir (O), simvastatin (S) and fenofibrate (F). (a) Pro-inflammatory cytokines in BALF and serum samples collected at 4, 6 and 8 days p.i. from inoculated mice treated with oseltamivir plus fenofibrate. (b) T-lymphocyte counts in peripheral blood collected at 4, 6 and 8 days p.i. from inoculated mice treated with oseltamivir plus fenofibrate. (c) Haematoxylin and eosin staining of lung tissues removed at 4, 6 and 8 days p.i. from inoculated mice treated with oseltamivir and fenofibrate, \(\times\)100 magnification. Values represent mean ± SD. *\(P<0.05\), #\(P<0.01\).
triple combinations of oseltamivir with statins and fibrates could benefit the survival rate of mice inoculated with lethal H5N1 virus (An et al., 2011). However, in this study, we found that simvastatin does not improve the effectiveness of oseltamivir alone following H7N9 virus infection in mice. We supposed that the discrepant results may depend on the virus used, because the H5N1 subtype could cause a more severe ‘cytokine storm’. A moderate subtype such as H1N1 is needed to verify this hypothesis. Belser et al. (2013) and Radigan et al. (2012) have reported that combination therapy with simvastatin and oseltamivir did not confer any additional benefit compared with the respective monotherapies when mice were infected with H1N1 or H3N2 viruses. It seems that when mice are infected with influenza subtypes that cannot induce pronounced leukopenia and lymphopenia, no added benefit to the efficacy of therapy will be observed when mice are treated with a combination of oseltamivir and simvastatin.

It is also worth noting that fibrates are used in clinical practice to treat hyperlipidaemia and other non-infectious, chronic inflammatory disorders, suggesting that combination therapy with oseltamivir plus fenofibrate should not present safety issues in the treatment of influenza infections (Belser et al., 2013; Grundy et al., 2005; The ACCORD Study Group, 2010).

Overall, our data suggest that treatment strategies for severely ill patients will most likely require a complex therapeutic approach to reduce viral replication and normalize aberrant immune responses. Combinations of oseltamivir and the immunomodulator fenofibrate should be considered for randomized controlled trials of treatments for patients infected with H7N9.

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