

# ***In vitro* evaluation of the antiviral activity of methylglyoxal against influenza B virus infection**

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**Summary** Influenza A and B virus infections are serious public health concerns globally. However, the concerns regarding influenza B infection have been underestimated. The currently used anti-influenza drugs have not provided equal efficacy for both influenza A and B viruses. Susceptibility to neuraminidase (NA) inhibitors has been observed to be lower for influenza B viruses than for influenza A viruses. Moreover, the emergence of resistance to anti-influenza drugs underscores the need to develop new drugs. Recently, we reported that methylglyoxal (MGO) suppressed influenza A virus replication in a strain-independent manner. Therefore, we hypothesize that MGO exhibits anti-influenza activity against B strains. This study aimed to evaluate the anti-influenza viral activity of MGO against influenza B strains by using Madin-Darby canine kidney (MDCK) cells. Several types of influenza B viruses were used to determine the activity of MGO. The susceptibilities of influenza A and B viruses to NA inhibitors were compared. MGO inhibited influenza B virus replication, with 50% inhibitory concentrations ranging from 23-140  $\mu$ M, which indicated greater sensitivity of influenza B viruses than influenza A viruses. Our results show that MGO has potent inhibitory activity against influenza B viruses, including NA inhibitor-resistant strains.

**Keywords:** Influenza virus, anti-influenza viral drug, methylglyoxal, neuraminidase inhibitors

## **1. Introduction**

Influenza viruses are enveloped, negative, single-stranded RNA viruses with eight segmented genomes belonging to the *Orthomyxoviridae* family. There are three distinct virus types, A, B, and C, distinguished according to their antigenicity to internal protein structures, nucleoprotein (NP) and matrix protein. Influenza A and B viruses are important human respiratory pathogens that cause epidemics with significant disease burden. Although concern regarding influenza B virus infection relative to influenza A in humans has been neglected in the past, recent studies have shown that influenza B infection

causes similar rates of mortality in some epidemic seasons, especially in children (1,2). Clinical reports also have shown that infection by influenza B viruses tends to induce lethal secondary bacterial infections and myocardial or neurological complications (3-6). Although influenza B viruses share similar fundamental structural features of this family, these have different characteristics from those of influenza A viruses; for example, the matrix BM2 has been found to have ion channel activity equal to that of influenza A viruses M2 (7). Moreover, the influenza A virus shows a more rapid rate of evolution than that of the influenza B virus, which lacks a wild animal reservoir (8).

During the last step of the virus life cycle, neuraminidase (NA) plays an important role in removal of sialic acid from cellular receptors recognized by hemagglutinin (HA), which results in the release of new progeny virions from infected cells. Because the HAs of neighboring virions recognize and bind to neuraminic acid residues, which cause self-aggregation of new progeny virions, release of new virions, therefore, requires the receptor-destroying activity of NA to cleave

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glycoconjugates between viral glycoproteins and host cell molecules (9,10). NA was chosen as a suitable drug target because it has a critical role in the influenza life cycle. Amino acid residues of the catalytic site or the framework of the enzyme are highly conserved in both influenza type A and B (11,12).

NA inhibitors (NAIs) were designed to be sialic acid analogues that potently and specifically inhibit influenza virus replication by competitively binding to the NA active site, which results in inhibition of cleavage of the cell surface and prevention of the release of newly formed virions (13). There are currently three Food and Drug Administration-approved drugs effective for influenza B virus worldwide, oseltamivir (Tamiflu<sup>®</sup>, Roche), zanamivir (Relenza<sup>®</sup>, GlaxoSmithKline), and peramivir (Rapivab<sup>®</sup>, Biocryst). Inhaled laninamivir (Inavir<sup>®</sup>, Daiichi-sankyo) is also approved in Japan.

Several studies have reported that NAIs may have a lower efficacy against influenza B viruses than against influenza A (9,14-16). *In vitro* studies also have shown that the 50% inhibitory concentrations (IC<sub>50</sub>) of oseltamivir were dramatically higher for influenza B than for influenza A viruses (17). The elevated IC<sub>50</sub> of oseltamivir for influenza B may result from the structure of NA protein that is less flexible than that of influenza A, which causes incomplete binding to the hydrophobic pocket of oseltamivir (18). The susceptibilities of NAIs have been considered to be dependent on the B lineage in the same manner as observed for different influenza A neuraminidase subtypes (14).

Natural products, such as microbial metabolites and medicinal plants, are promising as potentially effective and novel antiviral drugs. To date, several agents isolated from these natural products have been reported. We found that manuka honey exhibited the highest anti-influenza activity among tested honey samples (19). We recently reported that the  $\alpha$ -ketoaldehyde compound, methylglyoxal (MGO; molecular weight 72.06; Figure 1), which is present in extremely high concentrations in manuka honey (20), has potent inhibitory activity against multiple influenza A subtypes, including the oseltamivir-resistant influenza strain. The mechanism of MGO is thought to involve direct interaction of MGO on the virus surface and interference with the interactions between viruses and host cells. Because the mode of action of MGO is different from that of NAIs, MGO in combination with NAI could enhance the NAI activity for inhibition of influenza A virus replication (21).

In this study, we compared the anti-influenza viral

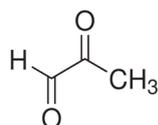


Figure 1. Chemical structure of MGO.

activity of MGO against influenza B viruses with that of other NAIs and evaluated its potential as a new universal antiviral agent against influenza viruses. In addition, we also compared the susceptibilities to NAIs of several strains of influenza B virus, including laboratory strains and clinically isolated samples from patients in Japan, with those of influenza A viruses reported previously (21). MGO exhibited a broad spectrum of inhibitory activity against influenza B viruses, not only against NAI-sensitive influenza virus B strains, but also against NAI-resistant influenza B strains. Moreover, influenza B viruses were found to be more sensitive to MGO than were influenza A viruses, as shown by lower IC<sub>50</sub> against influenza B viruses than against influenza A viruses.

## 2. Materials and Methods

### 2.1. Cells, viruses and chemicals

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (E-MEM) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) supplemented with 5% fetal bovine serum (FBS) purchased from Sigma-Aldrich (St. Louis, MO, USA) at 37°C in an atmosphere of 5% CO<sub>2</sub>. B/Lee/40 (ATCC<sup>®</sup> VR-1535<sup>™</sup>) and B/Brisbane/60/2008 were purchased from the American Tissue Culture collection (Manassas, Virginia, USA) and National Institute of Infectious Diseases (Tokyo, Japan), respectively. Influenza B clinical strains were isolated in our laboratory from clinical specimens collected from a hospital in Japan during 2014. Influenza virus B strains used in this study were propagated in MDCK cells. The 50% tissue culture infective dose (TCID<sub>50</sub>) of influenza virus was titrated by using MDCK cells. Oseltamivir, zanamivir, peramivir and laninamivir were purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland), GlaxoSmithKline PLC (Middlesex, UK), Biocryst, Inc. (Durham, NC, USA), and Daiichi Sankyo, Ltd. (Tokyo, Japan), respectively. Oseltamivir was dissolved in H<sub>2</sub>O to a concentration of 24 mM, and zanamivir and laninamivir were dissolved in dimethyl sulphoxide (CultureSure<sup>®</sup> DMSO) purchased from Wako Pure Chemical Industries, Ltd. to concentrations of 25 mM and 564.34 mM, respectively. Peramivir (30.5 mM) was directly used without dilution. All samples were maintained at -80°C. Approximately 40% MGO solution (in H<sub>2</sub>O) was purchased from Sigma-Aldrich and maintained at 4°C. Prior to performing experiments, MGO was diluted with E-MEM supplemented with 1% 100× vitamin solution (MEM-vitamin) purchased from Gibco<sup>®</sup> (Carlsbad, CA, USA).

### 2.2. Evaluation of anti-influenza viral and cytotoxic activities

The anti-influenza viral activities of MGO and NAIs

were evaluated as previously described (21), with slight modifications. For evaluation of anti-influenza viral activities, MDCK cells were typically seeded in 96-well plates at a density of  $3.0 \times 10^4$  cells/well in 100  $\mu$ L of MEM containing 10% FBS and incubated overnight. After washing with MEM-vitamin, 100  $\mu$ L of two-fold serially diluted samples (MGO or NA inhibitors in MEM-vitamin) were added. Cells were subsequently infected with 100  $\mu$ L of influenza virus B solution (B/Lee/40, B/Brisbane/60/2008, B2014/1, B2014/4, B2014/6, B2014/7 or B2014/8 in MEM-vitamin) equivalent to 100 TCID<sub>50</sub>. The culture plates were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for three days. Basically, infected cells were detached from the culture plate and viable cells still remained on the culture plate. Although CV staining could not distinguish between viable and dead cells, we performed CV staining after a 3-day incubation to assure that infected cells will be detached from culture plates. Moreover, we first evaluated the cytotoxicity of MGO using the WST-1 assay and CV staining and found that 50% cytotoxicity concentration (CC<sub>50</sub>) values evaluated by the WST-1 assay and CV staining assay were similar ( $1.6 \pm 0.4$  mM vs.  $1.4 \pm 0.4$  mM, respectively) (21). We decided to use CV staining for further evaluation. After incubation, the culture medium was removed, and 200  $\mu$ L of 70% ethanol was added for 5 min and then removed. Cells were stained with 200  $\mu$ L of 0.5% crystal violet (CV), purchased from Wako Pure Chemical Industries, Ltd., in water for 5 min. After washing with water and air drying, absorbance was measured at 560 nm using an Infinite M200 Tecan plate reader (Wako Pure Chemical Industries, Ltd.). The 50% virus inhibitory concentration (IC<sub>50</sub>) of each sample was calculated from dose-response curves, and the percentage of viable MDCK cells was plotted.

The criteria recommended by the World Health Organization (WHO) Antiviral Working Group for data interpretation of resistant phenotypes are related to fold changes in IC<sub>50</sub> values compared with those of the susceptible viruses, and the criteria for influenza B viruses are different from that of influenza A viruses. Influenza B was described as having 'normal inhibition' (< five-fold higher IC<sub>50</sub> than that of the susceptible reference virus), 'reduced inhibition, RI' (five-50-fold higher IC<sub>50</sub> than that of the susceptible reference virus) or 'highly reduced inhibition, HRI' (> 50-fold higher IC<sub>50</sub> than that of the susceptible reference virus) (22).

### 3. Results

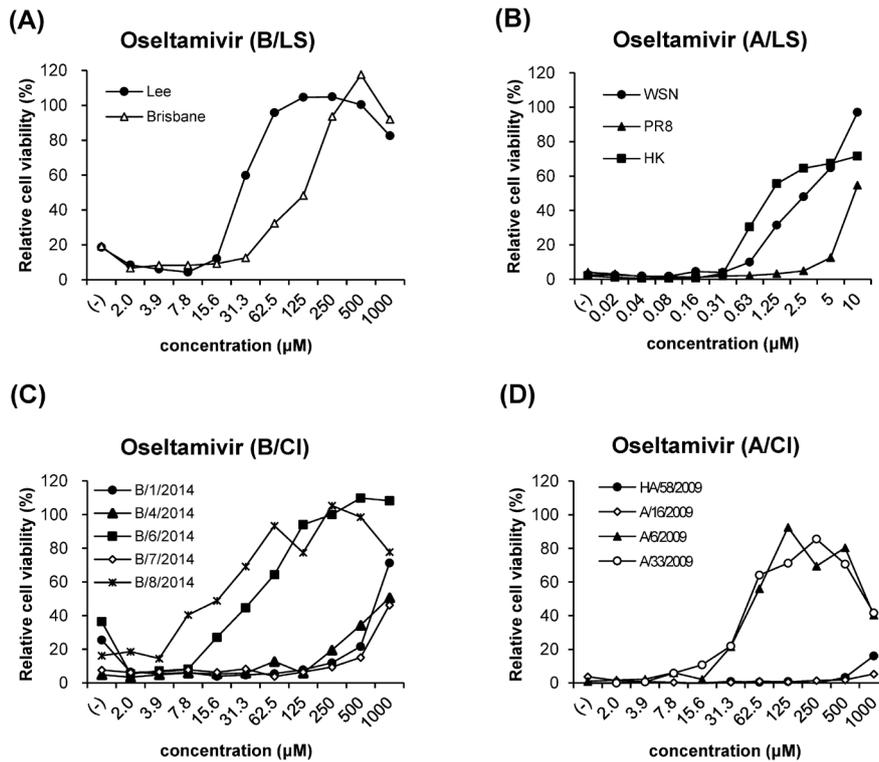
#### 3.1. Susceptibility to NAIs of influenza B viruses

We evaluated the antiviral activities against influenza B viruses of commercial NAIs to determine the susceptibility to each drug of several strains of

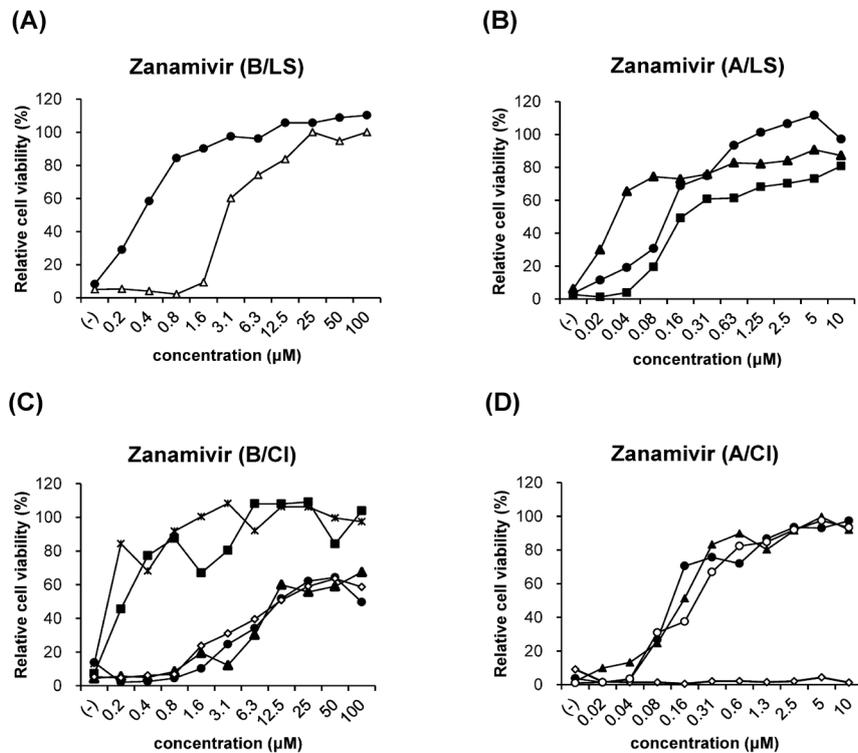
influenza B viruses. As reported in several studies, influenza B viruses exhibited the least susceptibility to NAIs (14,15,23). Our results also revealed that the mean IC<sub>50</sub> of oseltamivir against laboratory B strains (B/Lee/40 and B/Brisbane/60/2008) increased approximately ten-fold relative to the IC<sub>50</sub> values against laboratory A strains (A/WSN/33, A/PR/8, A/HK), as shown in Figures 2A and 2B. On the other hand, the efficacy of oseltamivir was not significantly different among the drug-sensitive clinical strains of A and B influenza viruses. For example, the IC<sub>50</sub> value of oseltamivir against drug-sensitive clinical influenza A viruses (A/2009/no.6 and A/2009/no.33) ranged from 36-39  $\mu$ M, which was not much different from that of oseltamivir against drug-sensitive clinical influenza B viruses (B/2014/6 and B/2014/8), which ranged from 11-33  $\mu$ M (Figures 2C, 2D and Table 1). The IC<sub>50</sub> of oseltamivir against drug-resistant influenza B viruses was also >500  $\mu$ M. Resistance to zanamivir in influenza B was not detected in either laboratory strains or clinical strains (Figures 3A and 3C); however, the susceptibilities of some clinically isolated influenza B viruses decreased relative to those of the influenza A viruses (Figures 3B and 3D) (Table 1). Interestingly, emergence of influenza B viruses with resistance to oseltamivir (Figure 2C) tended also to be resistant to laninamivir (Figure 4C) and peramivir (Figure 5C) (Table 1). Resistance of oseltamivir has also been shown to be cross-resistant to peramivir (24). We also demonstrated correlations between the drug resistance of oseltamivir and peramivir not just for influenza A viruses (Figures 2D and 5D) but also for influenza B viruses (Figures 2C and 5C). The susceptibility to peramivir of influenza B viruses dramatically decreased, as shown by the 20-fold and almost 70-fold increases in the mean IC<sub>50</sub> values for laboratory strains and drug-sensitive clinical strains, respectively (Table 1). On the basis of the criteria recommended by WHO, influenza B was determined to show reduced inhibition and highly reduced inhibition to peramivir for laboratory strains and drug-sensitive clinical strains, respectively.

#### 3.2. Antiviral activity of MGO against influenza virus B strains

In our previous study we evaluated the cytotoxicity of MGO and activity of MGO against influenza A viruses by using MDCK cells. We first evaluated the cytotoxicity of MGO using MDCK cells and determined a CC<sub>50</sub> value of  $1.4 \pm 0.4$  mM. Our study reported that MGO obviously suppressed influenza A virus replication in a strain-independent manner (21). However, the antiviral activity of MGO against influenza B types has not yet been evaluated. We first evaluated the inhibitory effect of MGO against influenza B viruses by using the same experimental



**Figure 2. Anti-influenza viral activity of oseltamivir.** Evaluation of the anti-influenza viral activity of oseltamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of oseltamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of oseltamivir (µM). (A), (B), (C), and (D) show the susceptibilities of oseltamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.



**Figure 3. Anti-influenza viral activity of zanamivir.** Evaluation of the anti-influenza viral activity of zanamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of zanamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of zanamivir (µM). (A), (B), (C), and (D) show the susceptibilities of zanamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

**Table 1. Efficacy of NAIs and MGO against various strains of IFV\***

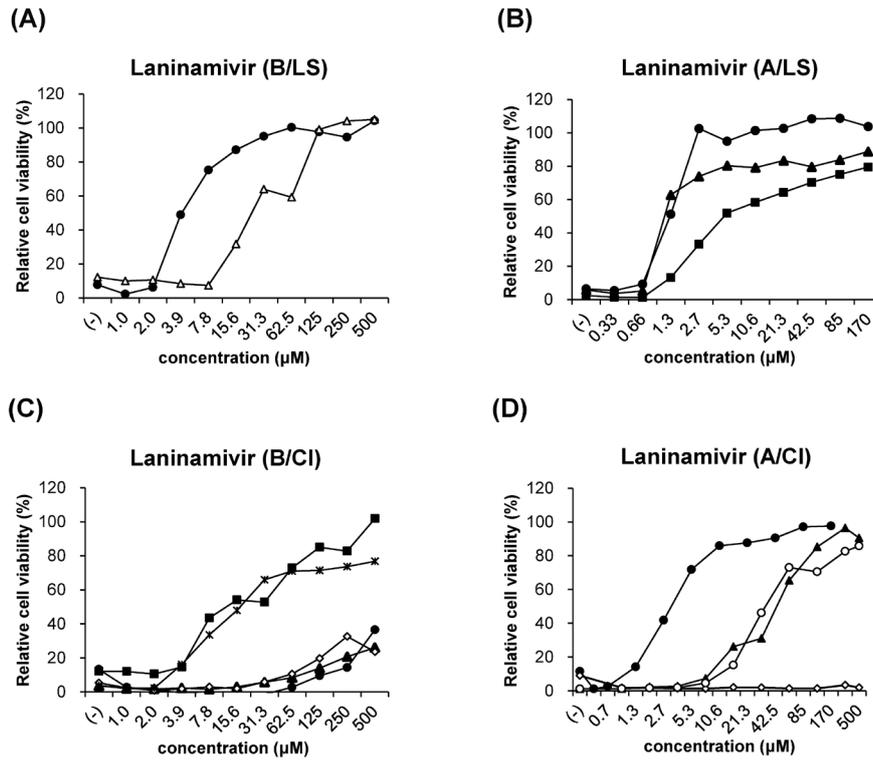
| Compound    | Virus strain | <sup>a</sup> IC <sub>50</sub> (μM) | Ave. (relative) | Virus strain    | IC <sub>50</sub> (μM) | Ave. ( <sup>b</sup> relative ratio) |               |                 |   |
|-------------|--------------|------------------------------------|-----------------|-----------------|-----------------------|-------------------------------------|---------------|-----------------|---|
| Oseltamivir | B/Lee/40     | <sup>c</sup> LS                    | 23 ± 3.5        | 49 (11.7)       | S                     | A/WSN (H1N1) LS                     | 2.5 ± 0.5     | 4.2 (1.0)       | S |
|             | B/Brisbane   | LS                                 | 75 ± 48         |                 | S                     | A/PR/8 (H1N1) LS                    | 9.4 ± 0.9     |                 | S |
|             | B/2014/6     | <sup>d</sup> CI                    | 33 ± 6.5        | 22 (5.2)        | S                     | A/HK (H3N2) LS                      | 0.7 ± 0.03    |                 | S |
|             | B/2014/8     | CI                                 | 11              |                 | S                     | A/no.6 (2009) CI                    | 36 ± 28       | 37.5 (8.9)      | S |
|             | B/2014/1     | CI                                 | > 810 ± 270     | > 926.7 (220.6) | R                     | A/no.33 (2009) CI                   | 39 ± 16       |                 | S |
|             | B/2014/4     | CI                                 | 970             |                 | R                     | A/HA-58 (2009) CI                   | 870           | > 935 (222.6)   | R |
|             | B/2014/7     | CI                                 | > 1000          |                 | R                     | A/no.16 (2009) CI                   | > 1000        |                 | R |
| Zanamivir   | B/Lee/40     | LS                                 | 0.32 ± 0.06     | 1.51 (10.1)     | S                     | A/WSN (H1N1) LS                     | 0.11 ± 0.02   | 0.15 (1.0)      | S |
|             | B/Brisbane   | LS                                 | 2.7 ± 5.2       |                 | S                     | A/PR/8 (H1N1) LS                    | 0.24 ± 0      |                 | S |
|             | B/2014/6     | CI                                 | 0.2             | 6.2 (41.3)      | S                     | A/HK (H3N2) LS                      | 0.11 ± 0.03   |                 | S |
|             | B/2014/8     | CI                                 | < 0.2           |                 | S                     | A/no.6 (2009) CI                    | 1.7 ± 0.8     | 1.34 (8.9)      | S |
|             | B/2014/1     | CI                                 | 11.7            |                 | S                     | A/no.33 (2009) CI                   | 2.2 ± 0.5     |                 | S |
|             | B/2014/4     | CI                                 | 9.9             |                 | S                     | A/HA-58 (2009) CI                   | 0.11 ± 0.02   |                 | S |
|             | B/2014/7     | CI                                 | 9.0             |                 | S                     | A/no.16 (2009) CI                   | > 100         | > 100 (666.7)   | R |
| Laninamivir | B/Lee/40     | LS                                 | 4.0 ± 0.21      | 13.5 (7.0)      | S                     | A/WSN (H1N1) LS                     | 1.2 ± 0.3     | 1.93 (1.0)      | S |
|             | B/Brisbane   | LS                                 | 23 ± 3.3        |                 | S                     | A/PR/8 (H1N1) LS                    | 1.6 ± 0.1     |                 | S |
|             | B/2014/6     | CI                                 | 12 ± 4.5        | 14.5 (7.5)      | S                     | A/HK (H3N2) LS                      | 3.0 ± 0.8     |                 | S |
|             | B/2014/8     | CI                                 | 17              |                 | S                     | A/no.6 (2009) CI                    | 48 ± 13       | 28.73 (14.9)    | S |
|             | B/2014/1     | CI                                 | > 500           | > 500 (259.1)   | R                     | A/no.33 (2009) CI                   | 35 ± 5.3      |                 | S |
|             | B/2014/4     | CI                                 | > 500           |                 | R                     | A/HA-58 (2009) CI                   | 3.2 ± 0.3     |                 | S |
|             | B/2014/7     | CI                                 | > 500           |                 | R                     | A/no.16 (2009) CI                   | > 500         | > 500 (259.1)   | R |
| Peramivir   | B/Lee/40     | LS                                 | 0.2 ± 0.06      | 0.52 (20)       | S                     | A/WSN (H1N1) LS                     | 0.011 ± 0     | 0.026 (1.0)     | S |
|             | B/Brisbane   | LS                                 | 0.84 ± 0.04     |                 | S                     | A/PR/8 (H1N1) LS                    | 0.061 ± 0     |                 | S |
|             | B/2014/6     | CI                                 | 2.8 ± 0.1       | 1.75 (67.3)     | S                     | A/HK (H3N2) LS                      | < 0.005       |                 | S |
|             | B/2014/8     | CI                                 | 0.7             |                 | S                     | A/no.6 (2009) CI                    | 0.12 ± 0.03   | 0.095 (3.6)     | S |
|             | B/2014/1     | CI                                 | 17 ± 3.5        | > 22.3 (859)    | R                     | A/no.33 (2009) CI                   | < 0.07 ± 0.03 |                 | S |
|             | B/2014/4     | CI                                 | > 25            |                 | R                     | A/HA-58 (2009) CI                   | > 2.5         | > 13.75 (528.8) | R |
|             | B/2014/7     | CI                                 | > 25            |                 | R                     | A/no.16 (2009) CI                   | > 25          |                 | R |
| MGO         | B/Lee/40     | LS                                 | 39 ± 10         | 31 (0.09)       | S                     | A/WSN (H1N1) LS                     | 240 ± 190     | 340 (1.0)       | S |
|             | B/Brisbane   | LS                                 | 23 ± 6.9        |                 | S                     | A/PR/8 (H1N1) LS                    | 360 ± 130     |                 | S |
|             | B/2014/6     | CI                                 | 48 ± 29         | 89 (0.26)       | S                     | A/HK (H3N2) LS                      | 420 ± 140     |                 | S |
|             | B/2014/8     | CI                                 | 140 ± 19        |                 | S                     | A/no.6 (2009) CI                    | 195 ± 79      | 234.5 (0.7)     | S |
|             | B/2014/1     | CI                                 | 110 ± 5.7       |                 | S                     | A/no.33 (2009) CI                   | 246 ± 2       |                 | S |
|             | B/2014/4     | CI                                 | 59              |                 | S                     | A/HA-58 (2009) CI                   | 250 ± 140     |                 | S |
|             | B/2014/7     | CI                                 | 88              |                 | S                     | A/no.16 (2009) CI                   | 247 ± 3.7     |                 | S |

\*Influenza virus strains were grouped into 3 groups, laboratory strain, drug-sensitive clinical strain, and drug-resistant clinical strain for influenza A and B viruses. Average IC<sub>50</sub> was calculated and represented in this table for each influenza group. Number in round bracket after average IC<sub>50</sub> represents relative ratio. <sup>a</sup>IC<sub>50</sub>: 50% inhibitory concentration. <sup>b</sup>relative ratio: The ratio average IC<sub>50</sub> against each influenza group was calculated relative to average IC<sub>50</sub> against influenza A laboratory strains. It was determined the differences of susceptibility of NAI against IFV in each group. <sup>c</sup>LS: laboratory strain. <sup>d</sup>CI: clinically isolated strain.

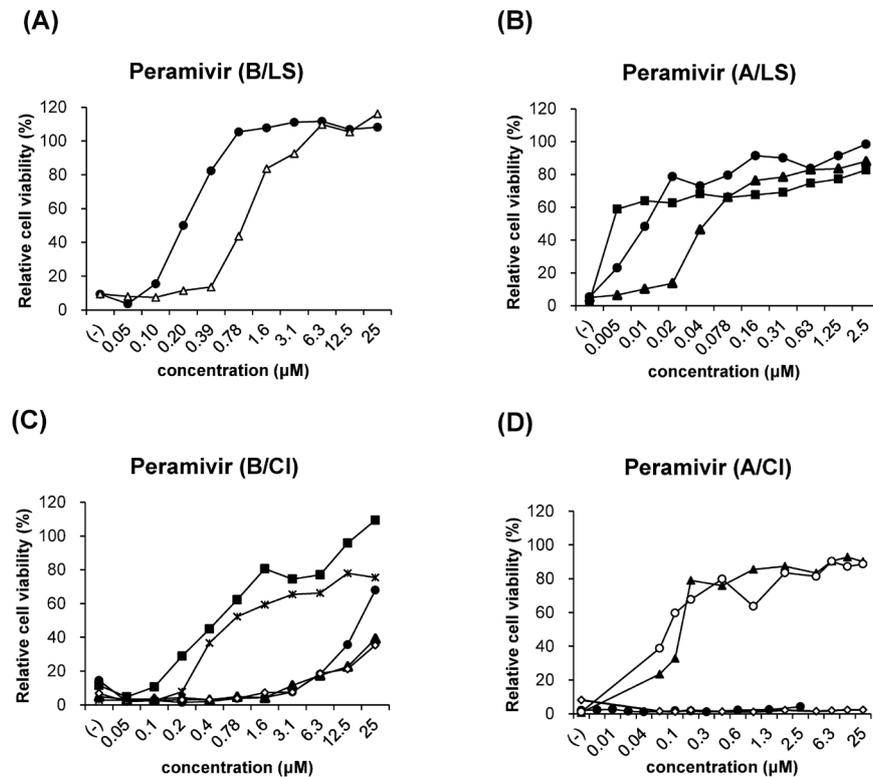
method used for evaluation of influenza A viruses to compare the anti-influenza viral activity of MGO between influenza types A and B, as shown in Figure 6. The viral cytopathic effect was suppressed in the presence of MGO in a dose-dependent manner for all influenza virus B strains, not only laboratory strains (Figure 6A), B/Lee/40 and B/Brisbane/60/2008, but also clinical strains (Figure 6C), B/2014/1, B/2014/4, B/2014/6, B/2014/7 and B/2014/8. The IC<sub>50</sub> values of MGO ranged from 23-140 μM against influenza B viruses and from 195-420 μM against influenza A viruses (Table 1), which indicated greater sensitivity of the influenza B viruses than the influenza A viruses (Figures 6B and 6D). Median IC<sub>50</sub> values of MGO against influenza B were 0.09-fold and 0.26-fold lower than those of influenza A viruses for laboratory strains and clinical strains, respectively.

#### 4. Discussion

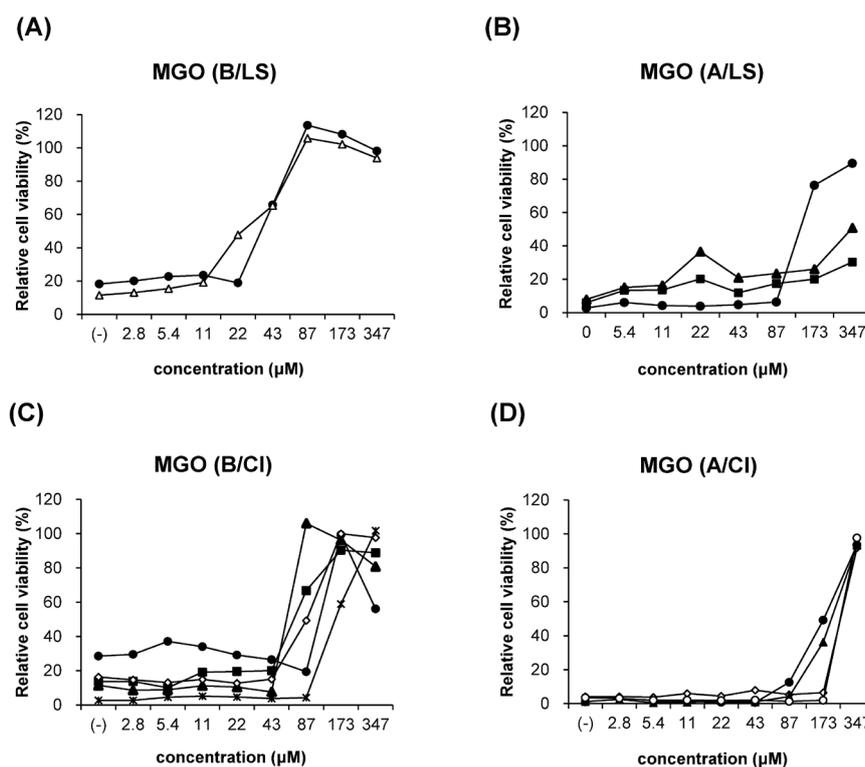
Influenza virus is a serious threat to human health. Influenza type A and B viruses share similar family characteristics, such as the negative single-strand RNA. However, there are also significant differences in epidemiology, evolutionary pattern, and host reservoir between the viruses (25,26). The emergence of influenza viruses with reduced susceptibility to NAIs has also been a critical issue recently, especially for influenza A and B viruses. Thus, the development of novel anti-influenza viral drugs is urgently required. Based on the finding of our recent report regarding the anti-influenza A viral activity of MGO (21), we hypothesised that MGO would also be effective against influenza B viruses in MDCK cells. Our present data indicate that MGO has antiviral activity against



**Figure 4. Anti-influenza viral activity of laninamivir.** Evaluation of the anti-influenza viral activity of laninamivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of laninamivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of laninamivir (µM). (A), (B), (C), and (D) show the susceptibilities of laninamivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.



**Figure 5. Anti-influenza viral activity of peramivir.** Evaluation of the anti-influenza viral activity of peramivir was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of peramivir and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of peramivir (µM). (A), (B), (C), and (D) show the susceptibilities of peramivir to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.



**Figure 6. Anti-influenza viral activity of MGO.** Evaluation of the anti-influenza viral activity of MGO was performed as described in the methods section. MDCK cells grown in 96-well plates were treated with serial dilutions of MGO and then infected with various strains of influenza viruses. Three days after infection, cells were fixed and stained with CV, and absorbance was measured using a plate reader. A graph was plotted showing relative CV staining (%), expressed as a percentage of uninfected cells and concentration of MGO ( $\mu\text{M}$ ). (A), (B), (C), and (D) show the susceptibilities of MGO to influenza B laboratory strains, influenza A laboratory strains, influenza B clinically isolated strains, and influenza A clinically isolated strains, respectively.

influenza B viruses, including influenza B virus with reduced susceptibility to NAIs (Figure 2 and Table 1). Interestingly, the susceptibility of MGO against influenza B viruses was higher than that against influenza A viruses, as suggested by the ten-fold and 3.8-fold reduced  $\text{IC}_{50}$  values for laboratory strains and clinical strains, respectively (Table 1).

Based on our finding that MGO shows anti-influenza activity in a strain-independent manner and can inhibit replication of influenza viruses with reduced susceptibility to NA inhibitors, the mechanism of MGO may not be related to interactions of HA or NA, in which mutation easily occurs. Some studies have reported that MGO showed antiviral activity against foot-and-mouth disease (27) and Newcastle disease virus (28) *via* interaction with viral RNA. The infectiousness of RNA isolated from MGO-treated virus was not infectious in subcutaneous inoculation of mice (27). The influenza virus polymerase does not possess a proof-reading function, so the virus rapidly adapts to certain selection pressures, thereby generating resistant viruses, especially if a viral protein is a drug target, such as an NAI. Because the extremely high mutation rate of influenza virus leads to the emergence of new virus strains that create a crucial problem for development of antiviral agents, the cellular cofactors that are necessary during influenza virus infection could be new targets

for drug development. Several studies have reported that during infection, influenza viruses activate the Raf/MEK/ERK-cascade and the transcription factor nuclear factor kappa B (NF- $\kappa\text{B}$ ) (29-32). The Raf/MEK/ERK-cascade is activated by influenza virus to support viral propagation, while inhibition of this cascade impairs function of the nuclear export protein, which results in accumulation of ribonucleoprotein complexes (RNPs) in the nucleus (33,34). Like the Raf/MEK/ERK-cascade, NF- $\kappa\text{B}$  is activated by influenza infection, then induces caspases, which subsequently supports the replication of influenza virus by enhancing RNP export (30,35). Molecules interfering with the NF- $\kappa\text{B}$  pathway, such as acetylsalicylic acid (ASA) (35), have been reported to have antiviral activity (31). Inhibition of cellular signalling of antiviral activity may be a novel function of new anti-influenza agents that target host-cell functions. Most importantly, no resistant virus variants have been shown to emerge in the presence of cellular pathway inhibitors, which suggests that influenza viruses cannot easily adapt to missing crucial cellular functions (34,35).

MGO has been reported to suppress tumor necrosis factor- $\alpha$ -induced NF- $\kappa\text{B}$  activation by inhibition of NF- $\kappa\text{B}$  DNA-binding and NF- $\kappa\text{B}$ -dependent reporter gene expression in a concentration-dependent manner (36), which is consistent with our data showing that MGO

can inhibit influenza replication in a dose-dependent manner. Similar to ASA, which blocks influenza virus by inhibiting NF- $\kappa$ B activity, MGO may inhibit influenza virus replication by interfering with NF- $\kappa$ B activation. Therefore, MGO can suppress influenza viral replication in a strain-independent manner.

The emergence of NAI-resistant influenza B viruses is a major public health concern worldwide. The emergence of NAI-resistant influenza B virus information is not well-understood, and concerns are often underestimated relative to those of influenza A viruses, although both viruses are regarded to cause significant disease burdens and to a similar degree. In Japan, the rate of NAIs used for clinical treatment have been found to be much higher than anywhere else in the world, and the use of NAIs has caused the spread of influenza B viruses with reduced susceptibility to NA inhibitors (16). Our results show that influenza A viruses are more susceptible to NAIs than influenza B viruses. The patterns observed for drug susceptibility were similar to those previously published (37). One possible explanation is that the binding affinities of HA protein of influenza B viruses and the sialic acid moiety are weaker than those of influenza A viruses (37,38). The problem of wild type influenza B viruses already having reduced susceptibility to NAIs relative to that of influenza A viruses is a concern because any further increase in IC<sub>50</sub> values due to mutations may induce complete loss of drug effectiveness in influenza B treatment or prophylaxis. Although the similarity of the amino acid composition of NAs between influenza A and B viruses is only 30% (39), the 19 amino acids at the catalytic site are highly conserved among all known influenza A and B NAs (40). Several clinical studies have reported that the locations of NA mutations differs among the NAIs used (26), and the locations of NA substitutions confer different levels of resistance among the NAIs used (37,41,42).

NA mutations of oseltamivir were observed at a higher rate than the rate for NA mutations of zanamivir. This phenomenon is because zanamivir is more similar to Neu5ac than is oseltamivir, so the binding of zanamivir to the NA active site is similar to natural substrate binding. Moreover, the rate of use of zanamivir is clinically lower than that of oseltamivir (43). Laninamivir is a long-acting derivative of zanamivir, which is administered as a single inhaled dose. The advantages of laninamivir are that it not only resides in the lung for many days (44) but also has slower dissociation than that of other NA inhibitors (45). Mutations at the location affecting the laninamivir dissociation rate can confer a dramatic resistance to laninamivir (46). Some studies have reported that mutations conferring zanamivir resistance also induce resistance to laninamivir with the loss of slow binding and/or faster dissociation (46), also relevant to our results (Table 1). Although our results do not show

any resistance of influenza B viruses to zanamivir, the median IC<sub>50</sub> of zanamivir against clinical strains resistant to other NA inhibitors (B/2014/1, B/2014/4, B/2014/7) was approximately 50-fold greater than those of clinical strains sensitive to other NA inhibitors (B/2014/6, B/2014/8). On the basis of the criteria recommended by WHO, B/2014/1, B/2014/4, and B/2014/7 were classified as showing highly reduced susceptibility to inhibition by zanamivir and all NAIs. Peramivir contains a guanidino group, as does zanamivir, and a hydrophobic group, as does oseltamivir; consequently, mutations affecting the activities of oseltamivir and zanamivir can also confer resistance to peramivir (47), and is supported by our results.

Although influenza A and B viruses belong to the family of Orthomyxoviridae, they possess distinct characteristics that are grouped into different types. Currently, several researchers are interested in influenza virus–host interactions. A difference in apoptosis profiles between influenza A and B strains has been reported recently (48). Influenza B viruses induce apoptosis earlier in infection, whereas influenza A viruses delay induction of apoptosis. Moreover, the transcription mechanisms of influenza A and B viruses enabled influenza B polymerase to recognize the cap structure in a manner different from that of influenza A polymerase, and the growth of influenza B viruses was more sensitive to the amount of cellular mRNA than was growth of influenza A viruses (49). These phenomena may explain our finding that MGO was more sensitive to influenza B viruses than to influenza A viruses and had a synergistic effect with NAIs only on influenza A viruses and not on influenza B viruses (data not shown).

In conclusion, our results show that MGO has potent inhibitory activity against influenza viruses A and B, including influenza viruses with reduced susceptibility to NAIs. Although influenza B viruses have been reported to be less sensitive to NA inhibitors than influenza A viruses, MGO shows higher sensitivity to influenza B, without observed resistance. Therefore, MGO has high potential as a universal anti-influenza agent, including potential for activity against resistant pandemic influenza viruses. A study to elucidate the mechanism of MGO and to determine whether it interacts with cellular mechanisms is in progress.

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