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Original Article

Efficacy of laninamivir octanoate in mice with advanced inflammation stage caused by infection of highly lethal influenza virus☆

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ABSTRACT

Four neuraminidase (NA) inhibitors and an RNA synthesis inhibitor were recently approved and are currently in clinical use for influenza. Among NA inhibitors, oseltamivir phosphate (OSE, Tamiflu®) and zanamivir are approved worldwide, whereas peramivir and laninamivir octanoate (LAN, Inavir®) are regionally approved for human use. Therefore, OSE has been used to treat infections of highly pathogenic influenza viruses, such as H5N1 and H7N9, which caused epidemic in southeast Asia and Egypt, and China, respectively. Generally, OSE is administered twice daily for 5 days by oral administration, and LAN once by inhalation for completing influenza therapy. In this study, we compared the efficacy of OSE and LAN administered according to the regimens in mice infected with highly lethal influenza viruses. The drugs were administered at the early and late stages of infection, which correspond to mild and severe inflammation in the lungs, respectively. Based on the drugs' regimens for human, a single administration of LAN at both stages of inflammation showed superior efficacy to repeated administration of OSE. LAN, as in OSE, could also be efficacious in treating severe influenza in humans.

1. Introduction

Influenza is a serious respiratory illness that can be debilitating and cause complications that lead to hospitalization and death, especially in elderly individuals. This respiratory disease is caused by influenza A and B viruses, which are pathogens that are highly contagious in humans. Influenza A viruses are classified into subtypes on the basis of the antigenicities of HA and NA molecules. To date, 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11) have been reported [1]. Seasonal influenza or influenza epidemics is caused by influenza A viruses, H1N1 and H3N2, and influenza B virus [2]. Every year, the global burden of influenza epidemics is believed to be 3.5 million cases of severe illness, and between 291,000 and 646,000 people worldwide die from seasonal influenza-related respiratory illness each year according to the new recent estimates [3]. Additionally, in the last 100 years, humans have experienced four influenza pandemics: the first in 1918 (H1N1), the second in 1957 (H2N2), the third in 1968 (H3N2), and the fourth in 2009 (H1N1pdm). Hundreds of thousands to tens of millions of deaths were reported from each pandemic worldwide [4–7]. Another possible concern is a pandemic caused by HPAI, such as H5N1 and/or H7N9 viruses, to which humans are immunologically naïve. Since 2003, H5N1 viruses have spread throughout Asia, Europe, and Africa with high morbidity and mortality among avian species, with occasional transmission with high mortality to humans. More than 50% mortality (454 deaths from 860 confirmed cases) was reported as of December 2018 [8]. The recent, more serious concern is the spread of an HPAI H7N9 virus in China since 2013 [9–11]. Approximately 40% mortality was reported as of September 2018 (615 deaths from 1567 confirmed cases) [12]. Although human-to-human transmission is rare, once H5N1 and/or H7N9 viruses acquire this ability, a devastating pandemic may be inevitable.

Abbreviations: NA, neuraminidase; OSE, oseltamivir phosphate; LAN, laninamivir octanoate; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; PFU, plaque forming unit; BALF, bronchoalveolar lavage flued; dpi, days postinfection; HD, human dose.

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Two countermeasures, vaccination and antivirals, are available to control seasonal human influenza. However, vaccination is an insufficient tool for both prophylaxis of seasonal influenza and against a pandemic influenza. Therefore, antivirals are an important tool for both the treatment and prophylaxis of influenza. Currently, three types of anti-influenza virus drugs are commercially available: M2 ion channel blockers (adamantine), NA inhibitors and RNA synthesis inhibitors.

NA inhibitors prevent efficient release of progeny viruses from host cells and have activities against influenza A and B viruses [13]. Two NA inhibitors, zanamivir (inhaled drug, 10 mg/dose; Relenza®) and oseltamivir phosphate (oral drug, 75 mg/dose; OSE, Tamiflu®), are currently licensed worldwide for human use. Both drugs are required to be administered twice daily for 5 days. In 2010, other NA inhibitors, peramivir (intravenous drip infusion drug, 300 mg/dose; Rapiacta®) and laninamivir octanoate (inhaled drug, 40 mg/dose; LAN, Inavir®), were licensed as influenza treatments by a single administration in Japan. In addition, RNA synthesis inhibitors, favipiravir and baloxavir marboxil (oral drugs), were also approved recently in Japan to treat influenza by inhibiting virus genome and proteins synthesis. LAN has also been approved as a chemoprophylaxis drug by a single inhalation. The reasons why a single dose of LAN is enough to treat and/or prevent influenza are well discussed in previous studies [14–16]. Briefly, LAN administered intranasally to mice is quickly converted to an active metabolite, laninamivir, by carboxylesterase in Golgi. As a result, the active metabolite may be retained for a long time in the cells of respiratory organs. Moreover, laninamivir has an ability of tight binding to the NAs of influenza viruses. By these unique characters of LAN in mice, the long-acting inhibitory effect of the compound was explained [16–18]. Finally, a single inhalation of LAN was confirmed to be efficacious as a treatment and prophylaxis of influenza in clinical studies [19–21].

It is difficult to obtain definitive evidence of the efficacy of NA inhibitors against HPAIV, such as H5N1 and H7N9, by clinical studies. However, the worldwide approved drugs OSE and zanamivir, both of which inhibit the NA activities of H5N1, are recommended by WHO for use against H5N1 influenza [22] and actually H5N1 infection and H7N9 infection as well have been used for treatment [9,11,24,25]. Laninamivir was reported to have inhibitory activities against the NAs of N1-9 subtypes [15], H5N1 [17], and H7N9 [23], including oseltamivir-resistant H5N1 with H275Y mutation [17] and H7N9 with R294K mutation [23]. Compared to OSE, LAN showed a higher efficacy in the epidemic season of H1N1 virus with H275Y mutation in clinical studies [19], as well as comparable efficacy to those of other 3NA inhibitors in post-marketing surveillance in Japan [26]. Therefore, LAN is a possible alternative as a treatment for HPAI H5N1 and/or H7N9 virus infection. Generally, HPAIV cause severe inflammation in the respiratory cells of mice [27,28], and humans [29,30]. LAN is suggested to incorporate the respiratory cells and convert to laninamivir to inhibit virus replication in mice [16]. However, it is unclear whether LAN uptake into inflamed cells and its conversion to laninamivir are elicited or not. To explore the possibility of LAN as a treatment for HPAIV infection, we evaluated its efficacy against advanced and mild stages of inflammation in mice infected with the mouse-adapted, highly lethal influenza virus A/PR/8/34 instead of HAPI viruses, and compared its efficacy with that of OSE.

2. Materials and methods

2.1. Compounds

LAN and OSE are obtained from Daiichi Sankyo Chemical Pharma Co., Ltd. and Daiichi Sankyo Co., Ltd., respectively.

2.2. Viruses and cells

The influenza virus A/Puerto Rico/8/34 (A/PR/8/34, H1N1) was kindly provided by Dr. Peter Palese (Mount Sinai School of Medicine, New York). Madin-Darby canine kidney cells (MDCK cells, ATCC CCL-34) were obtained from the American Type Culture Collection.

2.3. Animals

BALB/c mice (6 weeks old, female, specific-pathogen-free; Japan SLC, Inc.,) were kept in a controlled room throughout the experiments. The rearing conditions of the room were as follows: temperature of 23 ± 2 °C, relative humidity of 55 ± 10%, and 12-h light/dark cycle.

2.4. In vivo experiments

On day 0, the mice were anesthetized with 2.5% of isoflurane and infected intranasally with influenza virus at 500 PFU/mouse. The anesthetized mice were administered 50 μl (intranasally) or 200 μl (orally) of the compound dissolved in saline at the indicated dosages and times, as mentioned in each Figure. For the virus titration experiments, BALF from 6 mice was collected three times using 0.7 ml of phosphate buffered saline (PBS). After centrifuging the fluid at 1100 × *g* for 10 min at 4 °C, the supernatants were used for virus titration by the plaque assay. The precipitated fraction was suspended in PBS for counting the number of the white blood cells. All experimental procedures were performed in accordance with the rules and regulations of animal study practice at Kasai R&D Center of Kitasato Daiichi Sankyo Vaccine Co., Ltd.

2.5. Plaque assay

The samples were serially diluted 10-fold in minimum essential medium containing 0.2% BSA, 50 units/ml penicillin, and 50 μg/ml streptomycin. Confluently grown MDCK cells in 6-well plates were washed with PBS, and 200 μl of each diluted sample was added in duplicate. The cells were incubated at 37 °C under 5% CO_2 in an incubator for 1 h, and then washed with PBS. Next, 2.5 ml of modified eagle medium containing 0.2% of BSA, 25 mM of HEPES buffer, 0.01% of DEAE-Dextran, 1 μg/ml of trypsin, 0.001% of phenol red, and 0.6% of agar was added to the wells. The plates were placed in a CO_2 incubator for 2 days. After removing the agar medium, 0.1% crystal violet in 19% methanol was added to the wells to fix and stain the cells. The number of plaques on each well was counted.

2.6. Measurement of cytokines

TNFα in BALF was measured using the TNF alpha Mouse Uncoated ELISA Kit (Thermo Fisher Scientific).

2.7. Pathology

Mouse lungs fixed in 10% formalin buffer solution were stained with Hematoxylin and Eosin (HE), and with rabbit polyclonal antibody to influenza A, B (Takara Bio). Images were imported to a NanoZoomer-HT (Hamamatsu Photonics) for observation.

2.8. Statistical analysis

For experiments on the life-prolonging effect of LAN, statistical analysis was carried out by the Kaplan-Meier method and a log-rank test based on a joint ranking method. For the virus titration experiments, on the basis of virus titers (log_{10} PFU/lungs), a two-

way analysis of variance (ANOVA) was carried out for all titers on the titration days. The analyses were performed using SAS® System Release 9.02 for Windows® (SAS Institute, Inc.). *P* values of less than 0.05 were considered to be statistically significant. The symbols *, **, and *** for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, are also described in each figure.

3. Results

3.1. Early and advanced stages of lung inflammation in mice infected with A/PR/8/34 virus

The mice-adapted A/PR/8/34 virus we prepared causes severe lethal infection at 27 PFU, which is 50% of the mouse lethal dose, and all infected mice died at 6–8 dpi [14]. To assess the severity of inflammation in mice infected with A/PR/8/34 virus at 500 PFU, virus titers in the lungs, numbers of white blood cells (WBC) and neutrophils, and TNFα level in BALF were measured (Fig. 1A–C). In addition, the lungs were also stained with HE staining and immunostaining by antivirus antibody, and the images are shown in Fig. 1D. Virus titers plateau at approximately 10^7 PFU/BALF at 2–4 dpi and gradually decreased (Fig. 1A). At 3 dpi, body weight decreased approximately 10% from that before infection (data not shown). Moreover, the numbers of WBC and neutrophil, and TNFα level extremely increased at 3 dpi (Fig. 1B, C). Although virus antigens were observed partly in bronchial epithelial cells and the nearby alveolar cells, as observed by immunostaining at 1 dpi, cytopathic and desquamated bronchial epithelial cells and infiltration of inflammatory cells to the alveolar cells were observed by HE staining and virus antigens were widely spread in bronchial epithelial cells and the alveolar cells at 3 dpi (Fig. 1D). From 1 to 3 dpi, virus proliferation and the resulting inflammation occurred rapidly in mouse lungs. Therefore, we defined the infection stage at 1 dpi and 3 dpi as the early and advanced stages of inflammation, respectively.

3.2. Efficacy of NA inhibitors in the early stage of inflammation

LAN and OSE are approved to treat influenza in human adults by a single inhalation at 40 mg as a hydrate form, and by twice daily for 5 days at 98.5 mg as a phosphate form, respectively. Given that human body weight is 60 kg, the doses were calculated to be 0.69 mg/kg for LAN and 1.64 mg/kg for OSE. For the mouse model of early stage of inflammation, LAN was intranasally administered once at 1 dpi at 0.692 mg/kg [corresponding to 1 × human dose (1 × HD)] or 0.0692 mg/kg (0.1 × HD), and OSE was administered twice daily from 1 dpi at 39.4 mg/kg (24 × HD) or 3.94 mg/kg (2.4 × HD). All mice were administered saline intranasally or water orally according to the administration schedule of both compounds. Virus titers in the BALF of all mice administered with either compound were significantly decreased compared to that of the control mice, as analyzed by two-way ANOVA including all time points (Fig. 2). Single administration of LAN at 0.1 × HD showed a comparable reduction of virus titers to that of repeated administration of OSE at 2.4 and 24 × HD. Moreover, LAN at 1 × HD showed significant reduction of virus titers compared to that of repeated administration of OSE at 24 × HD (Fig. 2).

3.3. Efficacy of NA inhibitors in the advanced stage of inflammation

LAN was intranasally administered once at 3 dpi at 1 × HD or 0.1 × HD, and OSE was administered twice daily at 3 dpi at 24 × HD or 2.4 × HD. Virus titers in the BALF of control mice increased to about 10^7 PFU at 3 dpi and then decreased gradually. Virus titers in mice treated with either compound were significantly lower than those in the control after 4 dpi (Fig. 3A). By comparison between

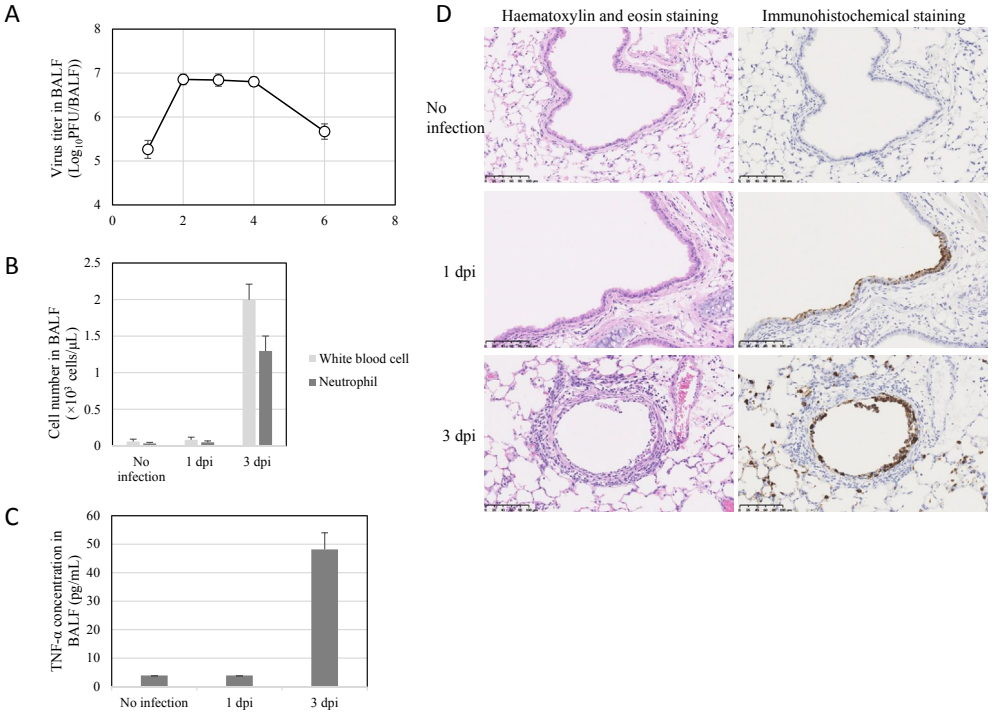

Fig. 1. Differences of inflammatory stages of mice at 1 and 3 days post-infection of influenza viruses. Virus titer changes in BALF of infected mice from 1 to 6 dpi (A). Cell numbers (B) and TNFα amounts (C) in BALF 1 and 3 dpi. Images of mouse lungs stained with hematoxylin and eosin and immunohistochemical staining at 1 and 3 dpi (D). Virus replication and inflammation in mouse lungs at 3 dpi (advanced stage of inflammation) were solidly progressive compared to those at 1 dpi (early stage of inflammation).

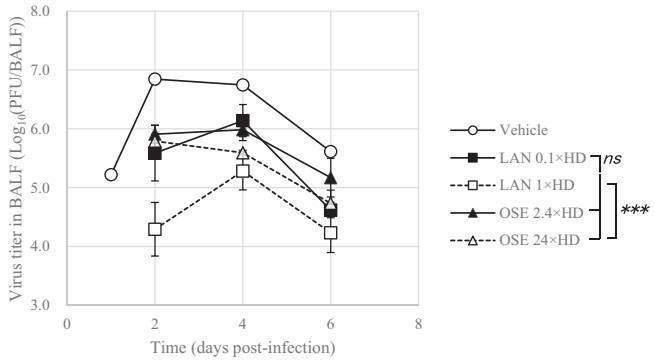

Fig. 2. Virus titers in BALF of mice administered LAN and OSE starting at 1 dpi, early stage of inflammation. Virus titers in BALF 2, 4, and 6 dpi were measured and analyzed between each group. Titers in all 4 groups treated with LAN and OSE were significantly decreased compared to those in the vehicle group ($p < 0.001$). Those of LAN at 0.1 × human dose (HD) were not significantly different to those of OSE at 24 × HD ($p = 0.94$). Those of LAN at 1 × HD were significantly reduced compared to those of OSE at 24 × HD ($p < 0.001$). ns: not significant. ***: $p < 0.001$.

the compounds, there were similar decreasing curves between 1 × HD of LAN and 24 × HD of OSE, and between 0.1 × HD of LAN and 2.4 × HD of OSE. There was also a significant titer decrease by 1 × HD of LAN compared to by 2.4 × HD of OSE (Fig. 3A).

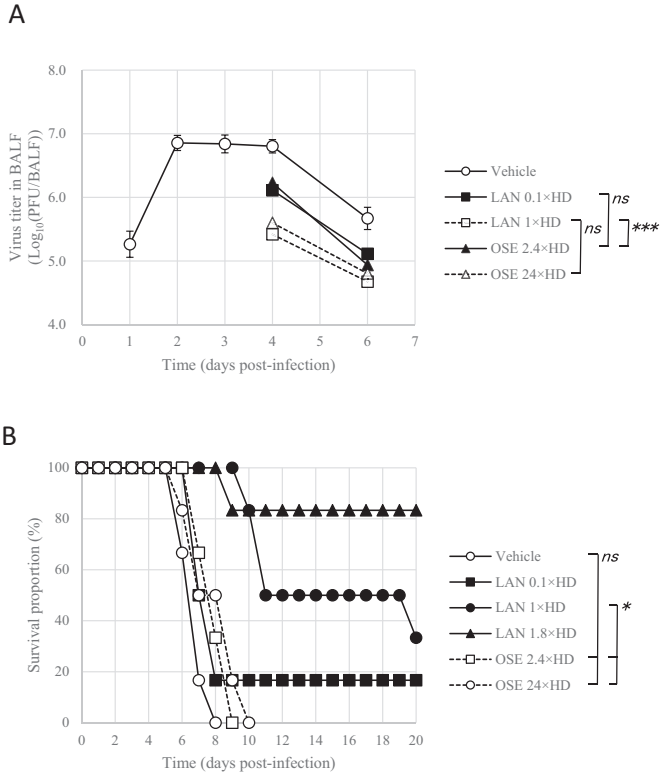

Fig. 3. Virus titers in BALF and survival of mice administered LAN and OSE starting at 3 dpi, advance stage of inflammation. Virus titers in BALF at 4 and 6 dpi were measured and analyzed between each group. Those of all 4 groups treated with LAN and OSE were significantly decreased compared to those of the vehicle group ($p < 0.001$). Those of LAN at 1 × human dose (HD) were significantly reduced compared to those of OSE at 2.4 × HD ($p < 0.001$) and not significantly different to those of OSE at 24 × HD ($p = 0.52$). (A). Survival of mice was monitored until 18 dpi. No significant difference in survival between the OSE and vehicle group was observed. Survival was significantly prolonged in the LAN group at 1 × HD compared to that in the OSE group at 24 × HD ($p < 0.041$). ns: not significant. *: $p < 0.05$.

Survival curves are provided in Fig. 3B. All mice repeatedly treated with OSE at 24 × HD or 2.4 × HD died at 10 dpi and there was only 1–2 days of prolonged survival compared to that in the control mice. On the contrary, 3 mice and 1 mouse out of 6 mice treated with LAN at 1 × HD and 0.1 × HD survived at 18 dpi, respectively. The significant survival-prolonging effect by LAN at 1 × HD was observed and compared to that by OSE at 24 × HD by Kaplan-Meier analysis. In addition, 5 of 6 mice survived owing to the single administration of LAN at 1.8 × HD, which is a maximum possible dose due to a restriction of its solubility in saline.

4. Discussion

At 1 dpi, ablation in tracheal epithelial cells of mice infected with influenza virus was not observed by HE staining and virus antigens were partly observed by immunostaining in the tracheal epithelial cells and the surrounding alveoli. At this early stage of infection, virus infection was restricted in the upper trachea without apparent cell deaths and virus spread did not progress deeply into the lungs. Therefore, we defined the stage at 1 dpi as early stage of inflammation. At 3 dpi, ablation and cytopathicity of tracheal epithelial cells, and infiltration of inflammatory cells, monocytes, and lymphocytes into alveolar cells around the trachea were observed by HE staining. Virus antigens were stained widely from the trachea to alveolar cells overlapping the inflammation area. As virus infection and the resulting inflammation in the lungs spread widely at this late stage after infection, we defined the stage at 3 dpi as advanced stage of inflammation.

In the early stage of inflammation, a single administration of LAN at a dose of 1 × HD showed a superior efficacy compared to repeated administration of OSE at a dose of 24 × HD in terms of virus reduction. These results supported previous data published elsewhere [14,15]. In the advanced stage of inflammation, a single administration of LAN at a dose of 1 × HD showed a similar virus reduction and significantly prolonged survival compared to repeated administration of OSE at a dose of 24 × HD. Pharmacokinetics of LAN and OSE are reported to be very much different from each other. In normal mice, LAN active form in lungs disappears slowly with t1/2 of 41.4 h [18], on the other hand, OSE active form in blood disappears quickly with t1/2 of 2.8 h [31]. As shown in Fig. 3, the difference between virus titers and survival rates at 1 × HD of LAN and 24 × HD of OSE are observed. This discrepancy may be due to a long retaining of active form of LAN in the target organ/cells and short half-life of OSE active form. These findings suggested that a single dose of LAN works both in the early and advanced stages of inflammation caused by virus infection.

LAN is suggested to require uptake into tracheal epithelial cells, which are target cells of influenza virus, generate its active form, laninamivir, in the cells, and remain there for a long time to exert an efficacy by a single administration [16,19]. The efficacy of a single dosing of LAN is believed to be due to similar pharmacokinetic features in both the early and advanced stages of inflammation. In the case of mild influenza virus infection, such as seasonal influenza, the therapeutic and prophylaxis efficacies of a single inhalation of LAN have been proved by clinical studies [19–21,32,33]. Considering NA inhibitory activities to H5N1 and H7N9 viruses [17,23], mechanism of long-action [16,18], animal study data in this report and clinical studies together, LAN can possibly be used as a therapy and prophylaxis for infection of HPAIV, such as H5N1 and H7N9 influenza virus. In addition, 2 RNA synthesis inhibitors, favipiravir and baloxavir marboxil, which have a different mechanism of action from LAN, are available for clinical use in Japan. Both showed additive efficacy by combinatorial therapy with NA inhibitors in mice infection model [34,35]. Combinatorial therapy of LAN with RNA synthesis inhibitors may be a good option to treat HAPIV infection."

Conflicts of interest

TT, KH and SK are employees of Daiichi Sankyo Co. Ltd. MY received speaker's honoraria from Daiichi Sankyo Co. Ltd. and MSD KK., and advisory contract fees from Daiichi Sankyo Co. Ltd. and Kitasato Daiichi Sankyo Vaccine Co. Ltd.

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