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Efficacy of a single intravenous administration of laninamivir (an active metabolite of laninamivir octanoate) in an influenza virus infection mouse model

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ABSTRACT

Laninamivir, a potent neuraminidase (NA) inhibitor, is an active metabolite of laninamivir octanoate (code name: CS-8958) which is a long acting NA inhibitor and is commercially available under the brand name Inavir in Japan to complete the treatment of influenza by a single inhalation. It is supposed that the long acting character is provided by the long retention of laninamivir in the respiratory tract after intranasal administration of laninamivir octanoate in mice and with stable binding of laninamivir to NA of various influenza viruses such as N1, N2 subtypes and NA of B virus. Peramivir, another NA inhibitor, is also approved in Japan as a single intravenous infusion. In spite of the quick disappearance of peramivir from the blood after injection, the reason treatment can be completed by a single administration is thought to be that peramivir showed stable binding to NA with N9 subtype. Therefore, the stable binder, laninamivir is possibly effective by a single intravenous administration in the mouse model infected with influenza viruses. A single intravenous administration of laninamivir and peramivir at 30 mg/kg significantly prolonged mice survival at a comparable level in the mouse lethal model infected with the A/PR/8/34 (H1N1) virus. Also, a single intravenous administration of laninamivir and peramivir significantly suppressed virus proliferation in the lungs of mice infected with influenza B virus. Thus, laninamivir may be effective by a single intravenous infusion in treating influenza, the same as peramivir.

1. Introduction

Influenza is a serious respiratory illness which can be debilitating and causes 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 for humans. Influenza A viruses are classified into subtypes on the basis of the antigenicities of hemagglutinin (HA) and neuraminidase (NA) molecules. To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been reported. Seasonal influenza or influenza epidemics are caused by influenza A virus H1N1 and H3N2 and influenza B virus (Wright and Webster, 2001); and every year the global burden of influenza epidemics was believed to be 3.5 million cases of severe illness and 300,000–500,000 deaths (Fiore et al., 2007), before the new pandemic in 2009.

Two countermeasures, vaccinations and treatment with antivirals, are available to control human influenza. Although vaccinations play an important role in influenza prophylaxis, they are an insufficient tool both for prophylaxis and against a pandemic virus. Therefore, antivirals are an important tool that may be used to mitigate influenza pandemics. Currently, two types of anti-influenza virus drugs are available: M2 ion channel blockers (adamantane) (Davies et al., 1964) and NA inhibitors. However, adamantane-resistant viruses readily emerge and are already prevalent worldwide among the seasonal influenza viruses such as the H1N1, the H1N1pdm09 and the H3N2 subtypes (Bright et al., 2005, 2006a,b CDC, 2009).

The NA inhibitors were discovered by a structure based drug design with the advent of the N2, N9 and influenza B NA structures (Colman et al., 1983; Varghese et al., 1983; Baker et al., 1987; Burmeister et al., 1992; von Itzstein et al., 1993, 1994; Von Itzstein, 2007). Two NA inhibitors, zanamivir and oseltamivir, are used worldwide for treatment of influenza. In addition, two more inhibitors, peramivir and laninamivir octanoate (code name CS-8958) have been used since 2010 in Japan. Zanamivir (inhalant, Relenza®) and oseltamivir (oral drug, Tamiflu®) are required twice daily for 5 days for treatment; on the other hand, for peramivir (injection

Abbreviations: MDCK, Madin–Darby canine kidney; FBS, fetal bovine serum; PBS, phosphate buffered saline; BSA, bovine serum albumin; pfu, plaque forming unit; MLD_{50}, 50% mouse lethal dose; hpi, hours postinfection; dpi, days postinfection; MRT, mean residence time; half-life, $t_{1/2}$; AUC, area under curve.

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drug, Rapiacta®) and laninamivir octanoate (inhalant, Inavir®), a single administration of the drug is sufficient for treatment.

Oral drugs and inhaled drugs have a limitation in clinical use for treatment of serious and/or complicated influenza patients. Administration of enterically administered oseltamivir can be challenging in the ICU where patients may have difficulty tolerating oral administration of capsules or where intestinal ileus may cause malabsorption (Ariano et al., 2010). Use of inhalants such as zanamivir and laninamivir octanoate are not suitable for patients on mechanical ventilation (Writing Committee of the WHO, 2010). As part of the emergency public health response, the Food and Drug Administration issued an Emergency Use Authorization (EUA) for the experimental intravenous peramivir in 2009. In these situations, an intravenous drug is quite essential for the treatment of serious and/or complicated influenza patients and, in fact, clinical studies of injection of oseltamivir and zanamivir are ongoing (Wathen et al., 2012, http://clinicaltrials.gov/ct2/show/NCT01231620?term=zanamivir&rank=1, US NIH last accessed April 9, 2013).

Under the EUA, peramivir was distributed by the Centers for Disease Control and Prevention (CDC) for treatment of hospitalized pediatric and adult patients with evidence of H1N1pdm09 virus infection and who (1) were not responding to antiviral therapy, or (2) the clinician deemed that enteral or inhaled delivery of NAIs was not dependable or feasible (http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm187980.htm, US FDA, last accessed April 9, 2013). Also, intravenous peramivir showed a significant shorter length of hospital stay for severe influenza patients infected with the H1N1pdm09 virus (Louie et al., 2012).

It has been reported that a single intravenous injection of peramivir showed efficacy similar to repeated oral administrations (twice daily for 5 days) of oseltamivir for influenza patients in the clinical study (Kohno et al., 2011). In spite of the quick disappearance of peramivir from the blood after intravenous injection (MRT = about 3 h, (http://www.info.pmda.go.jp/go/pack/6250405A1032_1_01/, JP PMDA, last accessed April 9, 2013), the reason to treatment can be completed by a single administration is thought to be that peramivir showed stable binding to NA with N9 subtype (Bantia et al., 2006).

Laninamivir octanoate (code name: CS-8958) shows an effective anti-influenza activity by a single intranasal administration in the mouse and ferret models infected with various influenza viruses (Yamashita et al., 2009; Kubo et al., 2010; Kiso et al., 2010). The reasons for the long actions are accounted for by the long retention of the active metabolite, laninamivir, in animal respiratory tracts after intranasal administration (Koyama et al., 2009) and the stable binding ability of laninamivir to NA of various influenza viruses (Kiso et al., 2010; Yamashita, 2010; Yamashita et al., 2010). In these previous reports, laninamivir is also indicated to be a strong binder to NAs of H1N1, H1N1pdm09, H3N2 and type B viruses. The binding stability of laninamivir is similar to peramivir and stronger than zanamivir for H1N1, H1N1pdm09; similar to peramivir and zanamivir for H3N2; however, it is stronger than both for type B virus. Therefore, the stable binder laninamivir is possibly effective by a single intravenous administration in the mouse model infected with influenza viruses. In this report, to evaluate the *in vivo* efficacy of intravenous laninamivir, we compare the efficacy by intravenous administration of laninamivir to peramivir and zanamivir in the mouse infection model with influenza viruses.

2. Materials and methods

2.1. Viruses and cells

The influenza viruses A/Puerto Rico/8/34 (A/PR/8/34, H1N1) and B/Malaysia/2506/2004 were provided by the National Institute of Infectious Diseases, Japan. MDCK cells were obtained from the American Type Culture Collection (ATCC CCL-34) and purchased from DS Pharma Biomedical Co., Ltd. (Japan); maintained in minimum essential medium containing 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were cultured in 5% CO_2 at 37 °C.

2.2. Compounds

Laninamivir octanoate, laninamivir and zanamivir were synthesized by Daiichi Sankyo Co., Ltd. Commercially available Rapiacta (Shionogi Co. Ltd.) was used as peramivir.

2.3. Inhibitory activity to influenza virus NA

The NA activity was measured according to the method described elsewhere. (Kubo et al., 2010). Briefly, the virus solution and the test compound were mixed in a 32.5 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH buffer (pH 6.5) containing 4 mM $CaCl_2$ and the mixture was preincubated at 37 °C for 30 min. Then, 4-MU-NANA (final concentration of 100 μM) was added and the mixture was incubated for another 60 min at 37 °C. The generated fluorescence was measured with the excitation at 360 nm and the emission at 460 nm, using a CytoFluor series 4000 instrument (Applied Biosystems Japan, Ltd.). The 50% inhibitory concentration (IC_{50}) was calculated by linear regression analysis using SAS System Release version 8.2 (SAS Institute Inc.) software.

2.4. Animal experiments

Female BALB/c mice (5–6 weeks old, specific pathogen free; Charles River Laboratories Japan, Inc.) were kept in a controlled room throughout the experiments. The rearing conditions of the room were as follows: a temperature of 20–26 °C, relative humidity of 55 ± 10% and a 12-h lighting cycle. The mice were anesthetized with isoflurane (Abbott Japan Co., Ltd.) and were intranasally infected with A/PR/8/34 at 100 pfu (3.7 MLD_{50}) or B/Malaysia/2506/2004 at 1000 pfu. For a single administration, 10 or 30 mg/kg of laninamivir, zanamivir, peramivir or saline was intravenously administered once at 13 hpi. Laninamivir octanoate at 0.14 mg/kg was intranasally administered once as well. For repeated administration, 3.0 mg/kg of these compounds or saline was intravenously administered once daily from 13 hpi for 5 days. Mice survival was monitored until 20 dpi. The virus titers in the mice lungs at 2, 3 and 6 dpi were measured by a plaque assay described below. All the experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

2.5. Plaque Assay

All the lungs excised from mice were homogenized in 1 ml of PBS containing 0.4% BSA, 50 units/ml penicillin and 50 μg/ml streptomycin (PBS-BSA/PS). After centrifuging the homogenates at 2200*g* for 5 min at 4 °C, the supernatants were diluted 10-fold serially with 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 the each diluted sample was added in duplicate. After the cells were incubated at 37 °C under 5% CO_2 in an incubator for 1 h, they were washed with PBS. Then, 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 the CO_2 incubator for 2 days. After removing the agar medium, 0.1% crystal violet cell 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. Statistical analysis

For the life prolonging effect experiments, the survival proportions (%) were calculated by the Kaplan–Meier method and a log-rank test based on a joint ranking method was carried out to compare the compound groups with the saline group. The statistical adjustment for multiple comparisons was calculated by the Bonferroni method, when necessary. For the virus titration experiments, based on the logarithms (log_{10} pfu/lungs) of the virus titers, a two-way analysis of variance (ANOVA) was carried out for all the titers on the titration days and a Dunnett test was carried out for the titers for each day. Here, the AUC was calculated by the trapezoidal rule based on the means of the virus titers (pfu/lungs) on Days 2, 3 and 6 at each dose. The analyses were performed using SAS® System Release 6.12 or 8.2 for Windows® (SAS Institute, Inc.). *P* values of less than 0.05 were considered to be statistically significant. *P* values are written in the text and figure legends. 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. Inhibitory activities of NA inhibitors to influenza viruses

The IC_{50}s of laninamivir, peramivir and zanamivir to A/PR/8/34 were 1.05, 0.618 and 1.43 nM, respectively and those of B/Malaysia/2506/2004 were 1.72 1.07 and 2.35 nM, respectively (Table 1). The IC_{50}s were comparable to each other among these regarding the two viruses.

Table 1
50% inhibitory concentration of NA inhibitors to the viruses used in this study.

	IC_{50} (nM)		
	Laninamivir	Peramivir	Zanamivir
A/PR/8/34	1.05	0.618	1.43
B/Malaysia/2506/2004	1.72	1.07	2.35

3.2. In vivo efficacy of a single intravenous administration of laninamivir, peramivir and zanamivir in the mouse/influenza A virus infection model

A single intravenous administration of laninamivir and peramivir at 30 mg/kg gave 80% mice survival at 20 dpi, although 30% survival of mice administered zanamivir was observed (Fig. 1). All three administration groups showed statistically significant life prolonging effects compared to the saline group ($P < 0.0001$: supplement data S1). Laninamivir and peramivir at 30 mg/kg showed statistically significant effects compared to zanamivir ($P = 0.0191$ and 0.0169, respectively: supplement data S1) but no statistical significance between laninamivir and peramivir was observed ($P < 0.9881$: supplement data S1). At 10 mg/kg, all three compounds had no significant benefit compared to saline (supplement data S1). Laninamivir octanoate administered intranasally once showed 60% survival only at 0.14 mg/kg with statistical significance ($P < 0.0001$: supplement data S1).

3.3. In vivo efficacy of repeated intravenous administrations of laninamivir, peramivir and zanamivir in the mouse/influenza A virus infection model

The repeated intravenous administrations of laninamivir and peramivir at 3 mg/kg once daily for 5 days showed a significant survival effect ($P < 0.0001$: supplement data S2) and more than 90% of mice administered laninamivir or peramivir survived at 20 dpi (Fig. 2). Only 20% of mice survived with zanamivir at 20 dpi without statistical significance ($P = 0.1944$: supplement data S2), as shown in Fig. 2. There was no statistical difference between laninamivir and peramivir ($P = 0.3173$: supplement data S2), and both compounds showed significant survival effects compared to zanamivir ($P < 0.0001$: supplement data S2).

3.4. Suppression of virus proliferation by a single intravenous administration of laninamivir, peramivir and zanamivir in the mouse/influenza B virus infection model

The mice infected with B/Malaysia/2506/2004 virus were treated once intravenously at 10 and 30 mg/kg of laninamivir,

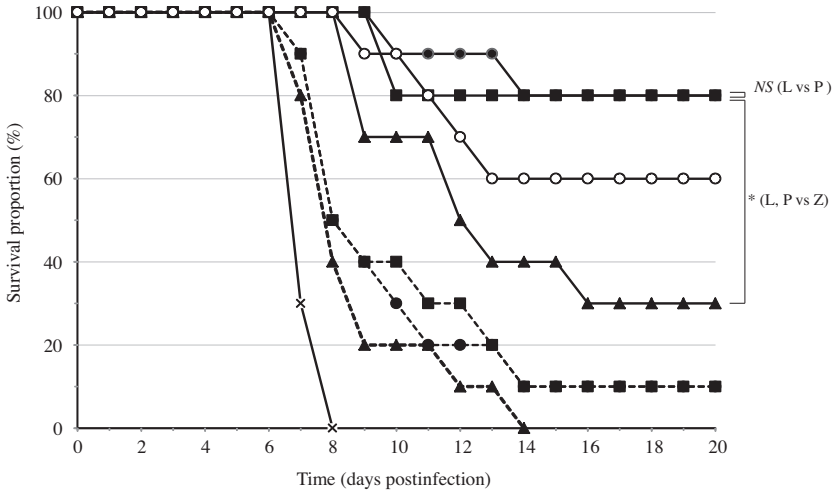

Fig. 1. *In vivo* efficacy of a single intravenous administration of laninamivir, peramivir and zanamivir in a mouse/influenza A virus infection model. Mice were infected with influenza A virus (A/PR/8/34, 100 pfu/mouse) on Day 0. Laninamivir (■), peramivir (●), zanamivir (▲) and saline (x) was intravenously administered once at 30 (solid line) and 10 (dotted line) mg/kg on 13 hpi. Laninamivir octanoate at 0.14 mg/kg (○) was intranasally administered once on 13 hpi. The number of surviving mice was monitored until 20 dpi. ($n = 10$). All groups intravenously administered NA inhibitor at 30 mg/kg showed a significant prolonged survival effect compared to the saline group ($P < 0.0001$), although no significant prolonged effect for all groups at 10 mg/kg. Both groups administered laninamivir and peramivir at 30 mg/kg showed significant prolonged effects compared to zanamivir at 30 mg/kg with $P = 0.0191$ and 0.0169, respectively. L: laninamivir, P: peramivir, Z: zanamivir.

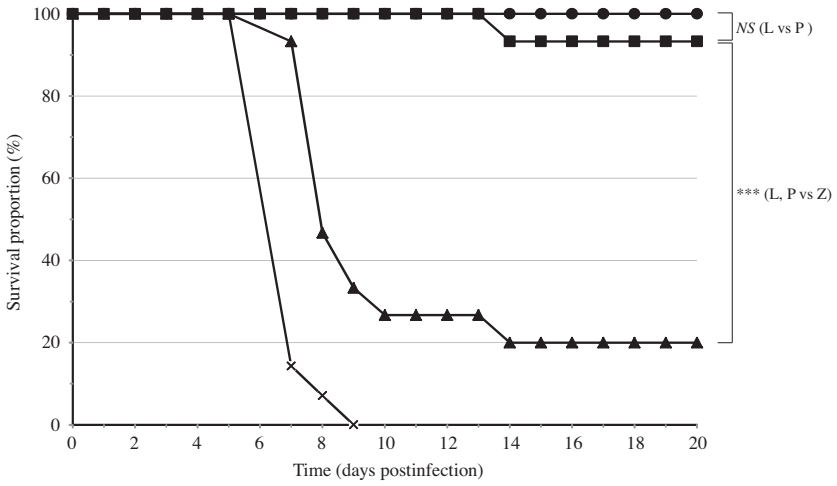

Fig. 2. *In vivo* efficacy of repeated intravenous administrations of laninamivir, peramivir and zanamivir in a mouse/influenza A virus infection model. Mice were infected with influenza A virus (A/PR/8/34, 100 pfu/mouse) on Day 0. Laninamivir (■), peramivir (●), zanamivir (▲) and saline (x) were intravenously administered at 3 mg/kg once daily for 5 days from 13 hpi. The number of surviving mice was monitored until 20 dpi. (n = 14–15). All groups intravenously administered NA inhibitor at 3 mg/kg showed a significant prolonged survival effect compared to the saline group (P < 0.0001). Laninamivir showed a significant survival effect compared to zanamivir with P < 0.0001 and no significant difference was observed between laninamivir and peramivir. NS: not significant. L: laninamivir, P: peramivir, Z: zanamivir.

zanamivir and peramivir on 13 hpi and virus titers in the lungs were measured on 2, 3 and 6 dpi (Fig. 3). In the control mice infected with B/Malaysia/2506/2004 at 1000 pfu, the viruses grew over 10^6 pfu/lungs at 3 dpi and then declined. All treated groups showed significant virus decrease compared to the saline group (P values were from <0.0001 to 0.0003: supplement data S3). Among the 30 mg/kg treated groups, laninamivir and peramivir suppressed the virus load significantly compared to the zanamivir group (P = 0.0058 and 0.0084, respectively: supplement data S3) and there was no statistical significance between laninamivir and peramivir (P = 0.2363: supplement data S3). As observed regarding good *in vivo* efficacy in the lethal infection model (Fig. 1), laninamivir octanoate again showed a good virus load reduction at much lower dosing compared to other NA inhibitors administered intravenously.

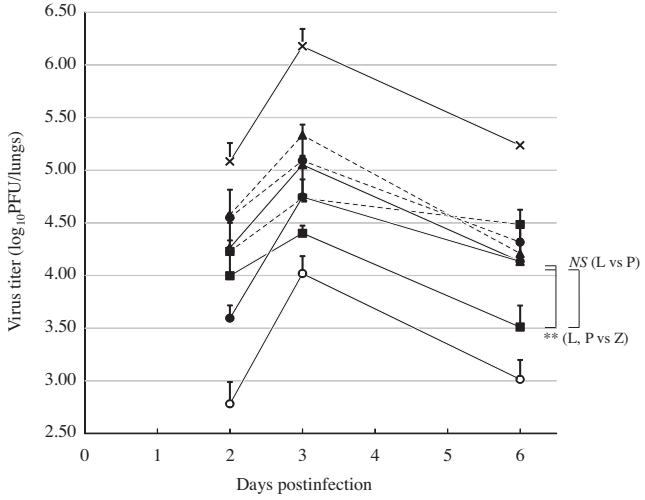

Fig. 3. Suppression of the virus load in lungs by a single intravenous administration of laninamivir, peramivir and zanamivir in a mouse/influenza B virus infection model. Mice were infected with influenza virus (B/Malaysia/2506/2004, 1000 pfu/mouse) on Day 0. Laninamivir (■), peramivir (●), zanamivir (▲) and saline (x) were intravenously administered at 30 (solid line) and 10 (dotted line) mg/kg on 13 hpi. Laninamivir octanoate at 0.47 mg/kg (○) was intranasally administered once on 13 hpi. The virus load in mice lungs on 2, 3 and 6 dpi was measured by the plaque assay. The log values of the virus titer (mean + SD, n = 3) are plotted. All groups intravenously administered NA inhibitor once at 30 mg/kg and laninamivir showed significant virus load suppression. Laninamivir suppressed the virus load significantly compared to zanamivir with P = 0.0058 and no significant difference was observed between laninamivir and peramivir. L: laninamivir, P: peramivir, Z: zanamivir.

4. Discussion

A single intravenous administration of laninamivir showed a similar life prolonging effect compared to peramivir in the lethal infection mouse model of influenza A virus. Both compounds showed a significantly higher survival rate than zanamivir (Fig. 1). Laninamivir octanoate administered intranasally showed a good *in vivo* efficacy at much lower dosing compared to other NA inhibitors administered intravenously (Figs. 1 and 3). It has been reported that laninamivir octanoate after intranasal administration quickly metabolized to laninamivir which was retained in the respiratory tracts, which are the target organ of influenza virus infection, for a long time with $t_{1/2}$ of 41.4 h (Koyama et al., 2009; Yamashita et al., 2010), and showed *in vivo* efficacy as a long-acting NA inhibitor (Yamashita et al., 2009; Kubo et al., 2010; Kiso et al., 2010). Intravenous laninamivir was not reported to be retained in lungs therefore it needed a high dose to show *in vivo* efficacy (Koyama et al., 2010). In the virus titration experiment, laninamivir and peramivir reduced the viral load in the lungs of mice infected with influenza B virus significantly lower than zanamivir by a single intravenous administration at 30 mg/kg.

The quick disappearance of laninamivir, peramivir and zanamivir from blood after intravenous administration of the compounds was reported. The $t_{1/2}$ of laninamivir and zanamivir were reported to be about 0.5 h in rats (Koyama et al., 2010) and 2.44 h in humans with normal renal function (Weller et al., 2013), respectively. Also, the MRT of peramivir after intravenous administration was reported to be about 3 h in humans (http://www.info.pmda.go.jp/go/pack/6250405A1032_1_01/, JP PMDA, last accessed April 9, 2013). In addition, there were no significant differences of the IC_{50}s to the influenza viruses used among the three compounds (Table 1). To explain, the differences of *in vivo* efficacy by a single intravenous administration of the compounds may be partly due to the differences of the binding stabilities as previously reported

(Bantia et al., 2006). In the report, Bantia et al. discussed that the efficacy of peramivir was superior to that of oseltamivir by a single administration in the influenza virus infection model of mice because the binding of peramivir to NA was more stable than that of oseltamivir.

Thus, it is clearly suggested that laninamivir shows an *in vivo* efficacy by a single intravenous administration similar to peramivir. These characteristics may be partly due to the stable binding to NAs of influenza A and B viruses. Although the binding stabilities of peramivir and zanamivir to NA of B virus were almost identical (Kiso et al., 2010; Yamashita, 2010), the reduction of the viral load in the lungs of mice infected with the B virus was significantly larger for peramivir than zanamivir. The discrepancy is unclear. The IC_{50}s of the compounds are comparable to each other, however the small difference between the two cannot be ruled out completely to explain the discrepancy (Table 1). The most likely explanation may be the pharmacokinetic difference between the compounds after intravenous administration. Further experiments are necessary.

Laninamivir is possibly effective by a single injection for normal influenza and by a repeated injection for severe influenza like peramivir. Peramivir shows a cross resistance with oseltamivir to the A/H1N1 virus with H274Y mutation (Nguyen et al., 2010) which was the most concerning mutant to cause worldwide epidemic until the 2008/09 season (http://www.who.int/influenza/resources/documents/H1N1webupdate20090318_ed_ns.pdf, WHO, last accessed April 9, 2013), where A/H1N1pdm09 with H274Y mutation had already begun to appear (Leang et al., 2013). Fortunately, the H274Y mutant is susceptible to laninamivir and zanamivir (Nguyen et al., 2010). These compounds should be another option to treat severe influenza patients by injection. However, as reported regarding some differences of IC_{50}s to the recent virus isolates among the NA inhibitors including the compounds used in this report (Ikematsu et al., 2012), the experiments using the recent isolates will be necessary to confirm *in vivo* efficacy.

Laninamivir octanoate (Inavir®) at 40 mg/body (adults) and 20 mg/body (children) has been used in humans to treat influenza by a single inhalation as a long-acting NA inhibitor due to the characteristics of long retention in the respiratory tract and stable binding to NA (Yamashita et al., 2010). However, as its active metabolite, laninamivir did not have the long retention characteristics (Koyama et al., 2010), where the dosing of intravenous laninamivir may be the same as or comparable to that of peramivir (Rapiacta®) which is a single dose of 300 mg/body (adults) or 10 mg/kg (children) for regular use to treat influenza. To find a proper dosing of intravenous laninamivir, clinical studies are essential. Inhalants such as zanamivir and laninamivir octanoate are beneficial for usual influenza patients but there is a limitation at clinical use for treatment of serious/complicated influenza patients, and young infant children patients who have difficulty inhaling. In contrast, an intravenous drug is quite suitable for such patients rather than inhalants. Injectable laninamivir will provide another option as a therapeutic agent because it maintains effectiveness against oseltamivir/peramivir resistant mutants with H274Y mutation (Yamashita et al., 2009; Nguyen et al., 2010).

Conflict of interest

MK, SK, TT and MY are employees of Daiichi Sankyo Co. Ltd., and MT and ST work for the company as temporary staff.

Other

A part of the data in this report has been published at the ESWI conference in Malta, in 2010.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.08.004.

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