Antiviral Research 109 (2014) 110–115

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

The relationship between *in vivo* antiviral activity and pharmacokinetic parameters of peramivir in influenza virus infection model in mice

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ARTICLE INFO

Article history:
Received 11 February 2014
Revised 18 June 2014
Accepted 24 June 2014
Available online 2 July 2014

Keywords:
Influenza
Neuraminidase inhibitors
Peramivir
PK/PD analysis

ABSTRACT

The purpose of this study was to investigate the relationship between pharmacokinetic (PK) parameters of intravenous (IV) peramivir and *in vivo* antiviral activity pharmacodynamic (PD) outcomes in a mouse model of influenza virus infection. Peramivir was administrated to mice in three dosing schedules; once, twice and four times after infection of A/WS/33 (H1N1). The survival rate at day 14 after virus infection was employed as the antiviral activity outcome for analysis. The relationship between day 14 survival and PK parameters, including area under the concentration–time curve (AUC), maximum concentration (C_{max}) and time that drug concentration exceeds IC_{95} ($T_{>IC95}$), was estimated using a logistic regression model, and model fitness was evaluated by calculation of the Akaike information criterion (AIC) index. The AIC indices of AUC, C_{max} and $T_{>IC95}$ were about 114, 151 and 124, respectively. The AIC of AUC and $T_{>IC95}$ were smaller than that of C_{max}. Therefore, both AUC and $T_{>IC95}$ were the PK parameters that correlated best with the antiviral activity of peramivir IV against influenza virus infection in mice.

1. Introduction

Influenza is an acute viral respiratory illness that causes high morbidity and mortality globally (Ruuskanen et al., 2011, Thompson et al., 2003). Three circulating sub(types), type A/H1N1, type A/H3N2 and type B, are well known as the human pathogens that cause massive and rapidly evolving epidemics globally in every winter season. Among these three influenza virus types, the dominant seasonal strain can vary markedly from year to year. In this century, pandemic influenza A/H1N1 spread rapidly beginning in the spring of 2009, resulting in significant morbidity and mortality (Shrestha et al., 2011). The occurrence of influenza epidemics or pandemics is still a major public health concern through the world. Even when the dominant circulating influenza strain persists over several seasons, yearly epidemics of influenza virus can be caused by mutations in the amino acids of two glycoproteins, hemagglutinin and neuraminidase (NA). This antigenic drift reduces the protective effect of humoral and cellular immunity resulting from prior influenza infections; consequently, seasonal influenza vaccines do not work completely at the present time (Belshe, 2005; Johansson et al., 2007).

Therefore, antiviral agents are of utmost important as countermeasures to influenza virus infection. The approved antiviral agents for treatment and prophylaxis of influenza infection are M2 channel and NA inhibitors (NAIs, Feng et al., 2012). At present, NAIs, which have potent activity against influenza A and B viruses, are the first choice antiviral agents. Amino acid substitutions in NA can lead to the resistance to NAI (Hurt et al., 2012; Nguyen et al.,

Abbreviations: PK, pharmacokinetic; IV, intravenous; PD, pharmacodynamic; AUC, area under the concentration–time curve; C_{max}, maximum concentration; IC_{50}, 50% inhibitory concentration; IC_{95}, 95% inhibitory concentration; $T_{>IC95}$, time that drug concentration exceeds IC_{95}; AIC, Akaike information criterion; NA, neuraminidase; NAIs, NA inhibitors; MDCK, Madin–Darby canine kidney; MEM, minimum essential medium; MUNANA, 2′-(4-methylumbelliferyl)-α-D-N-acethylneuraminic acid.

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http://dx.doi.org/10.1016/j.antiviral.2014.06.016
0166-3542/

2012; Samson et al., 2013) resulting in less effective treatment (Kawai et al., 2009; Saito et al., 2010). Peramivir, which was approved in Japan, South Korea and China and applied to FDA in US, is the only intravenous formulation among approved NAIs (Shetty and Peek, 2012). Peramivir has demonstrated potent antiviral activity against both type A and type B influenza viruses, including pandemic influenza A/H1N1 and avian influenza viruses, such as H5N1 or H9N2 strains (Govorkova et al., 2001; Nguyen et al., 2010). In some clinical studies, a single intravenous dose of peramivir demonstrated excellent clinical efficacy for influenza virus infection and good tolerability (Kohno et al., 2011a, 2011b; Sugaya et al., 2012).

Recently, interest in optimization of dosing regimens for medicinal products to achieve maximum efficacy in the clinical setting has focused on understanding the relationship between pharmacokinetic (PK) and pharmacodynamic (PD). In the infectious diseases field, a large number of PK/PD studies for antibacterial agents have been conducted using data from both non-clinical studies and clinical trials (Ambrose et al., 2007; Jacobs, 2001). Non-clinical PK/PD studies are important not only to evaluate the regimens administrated in the clinical setting, but also to reduce the risk and cost for development of new antimicrobial agents (Andes and Craig, 2002; Craig, 2001). Several non-clinical PK/PD studies for antiviral agents, such as anti-retroviral agents, anti-hepatitis agents and influenza NAIs, have also been conducted (Craig, 1998; Schmidt et al., 2008). These PK/PD studies of antiviral agents mainly used an *in vitro* hollow-fiber infection model system (McSharry et al., 2009; McSharry et al., 2011), while few studies of antiviral PK/PD have been reported using *in vivo* infectious models (Schmidt et al., 2008). Therefore, in order to establish a firmer basis for PK/PD relationships of antiviral agents, additional *in vivo* infectious PK/PD studies are warranted. Moreover, one clinical PK/PD study and one non-clinical PK/PD study for oral peramivir were reported (Drusano et al., 2001; Iyer et al., 2002). But the correlation among these two studies was obscure and the PK/PD study of intravenous peramivir has never been conducted.

The purpose of this study was to investigate the relationships between *in vivo* antiviral activity pharmacodynamic outcomes and PK parameters of intravenous peramivir.

2. Materials and methods

2.1. Compounds

Peramivir trihydrate (BCX-1812, RWJ-270201) was synthesized by BioCryst Pharmaceuticals (Birmingham, AL). Oseltamivir carboxylic acid was synthesized by Shionogi & Co., Ltd (Japan).

2.2. Viruses and cells

The influenza virus A/WS/33 (ATCC®VR-825™, H1N1) was obtained from American Type Culture Collection (Manassas, VA) and the others were obtained from Hokkaido University. Madin–Darby canine kidney (MDCK) cells were obtained from the European Collection of Cell Culture (Salisbury, UK) and were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum in humidified atmosphere of 5% CO_2 at 37 °C.

2.3. NA inhibition assay

NA inhibition assay was conducted as described before (Gubareva et al., 2002). 2′-(4-methylumbelliferyl)-α-D-N-acethylneuraminic acid (MUNANA, Sigma–Aldrich) was used as a substrate. Various concentrations of peramivir, appropriate concentration of viruses, which were inactivated by NP-40 (final concentration: 0.1%) and were determined from NA activity assay, and 10 mM of MUNANA were mixed in a black opaque 96-well plate. These mixtures were incubated for 30 min at 37 °C. Following addition of the stop solution the fluorometric intensity of 4-methylumbelliferon released from MUNANA was measured. The percent inhibition at each concentration of peramivir was determined and the 95% inhibitory concentration was calculated by non-linear sigmoid curve fitting. Each test was conducted in triplicate.

2.4. Antiviral study in mice

Specific pathogen-free 6 week-old female BALB/c mice (Charles River Laboratories Japan) were used in challenge experiments. All mouse studies were conducted under applicable laws and guidelines and with the approval of the Shionogi Animal Care and Use Committee. Mice were inoculated with 5×10^3 50% of tissue culture infectious dose of A/WS/33 (H1N1) in 100 µl intranasally under anesthesia by intramuscular administration of ketamine, xylazine and saline mixture. Intravenous administration of peramivir was started 48 h after virus infection. A total of 18 types of dosing regimens (6 types of amount of dosages and 3 types of dose frequencies) were administrated or total daily doses of peramivir were 0.25, 0.5, 1, 2, 4 and 8 mg/kg/day and all doses were administrated on three schedules: once, twice and four times within 12 h (Fig. 1). There were 10 mice in each treatment group and 20 mice in the saline control group. Mice were monitored daily for mortality in 14 days. The survival rate at day 14 was defined as antiviral activity for investigation of the correlation to PK parameter.

2.5. Pharmacokinetic evaluation in infected mice

Single dose of peramivir (0.4, 2 and 10 mg/kg) was administered to infected mice intravenously 48 h after inoculation of virus (Fig. 1). The blood was collected from heart under anesthesia. Plasma was prepared by immediate addition of heparin sodium and centrifugation of whole blood at 3,000 rpm for 10 min at 4 °C

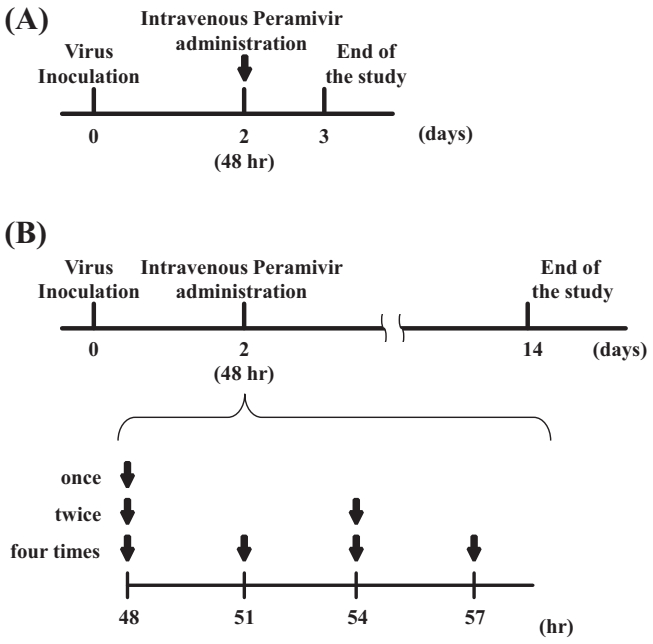

Fig. 1. The schematic illustration of the schedule for PK evaluation (A) and antiviral study (B). The results of PK evaluation represent in Fig. 2, and the results of antiviral study represent in Fig. 3.

Table 1
The IC_{95} values of peramivir trihydrate and oseltamivir carboxylic acid against neuraminidase derived from various influenza virus strains.

Virus	Subtype	IC_{95} (nM)[a]		IC_{50} (nM)[a]	
		Peramivir trihydrate	Oseltamivir carboxylate	Peramivir trihydrate	Oseltamivir carboxylate
A/WS/33	H1N1	5.94	18.64	0.31	0.98
A/PR/8/34	H1N1	9.56	26.67	0.50	1.40
A/duck/Hokkaido/W159/2006	H6N1	11.76	39.80	0.62	2.09
A/Singapore/1/57	H2N2	17.80	12.60	0.94	0.70
A/duck/Hokkaido/13/2000	H9N2	21.23	16.30	1.12	0.86
A/duck/Hokkaido/84/2002	H5N3	17.19	19.65	0.90	1.03
A/duck/Hokkaido/17/2001	H2N3	13.20	18.77	0.69	0.99
A/turkey/Ontario/6118/1968	H8N4	17.20	23.16	0.91	1.22
A/duck/Hokkaido/18/2000	H10N4	9.62	23.20	0.51	1.22
A/duck/Alberta/60/1976	H12N5	5.61	19.45	0.30	1.02
A/duck/Hokkaido/1058/2001	H4N5	4.64	18.80	0.24	0.99
A/duck/England/1/1956	H11N6	15.54	23.03	0.82	1.21
A/duck/Hokkaido/W186/2006	H13N6	13.04	22.70	0.69	1.19
A/seal/Massachusetts/1/1980	H7N7	16.23	16.28	0.85	0.86
A/chicken/Germany/N/1949	H10N7	22.42	46.72	1.18	2.46
A/duck/Ukraine/1/1963	H3N8	7.39	64.31	0.39	3.38
A/duck/Hokkaido/228/2003	H6N8	6.59	41.65	0.35	2.19
A/duck/Memphis/546/1974	H11N9	8.56	22.34	0.45	1.18
A/duck/Hokkaido/W245/2004	H11N9	7.40	21.52	0.39	1.13

[a] A mean value of IC_{90} or IC_{50} was calculated from two or three independent experiments.

and further processed by addition of methanol and repeat centrifugation. Peramivir concentration was determined using a validated liquid chromatography/tandem mass spectrometry (LC/MS–MS) method at Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan) as described previously (Kohno et al., 2010). A two-compartment model was fitted to plasma concentration data and model parameters estimated using WinNonlin (Ver. 5.01 or greater, Pharsight Corporation).

2.6. PK/PD analysis

Area under the concentration–time curve (AUC), peak concentration (C_{max}) and time that drug concentration exceeded IC_{95} ($T_{>IC95}$), were estimated based on the model parameters. To investigate the relationship between antiviral activity and PK parameters, a logistic regression model was applied. The dependent variable in the model was the occurrence of death 14 days after inoculation of influenza virus and the independent variable was each PK parameter (SAS Ver. 8.02 or greater for windows): Pr (death) = p(β0 + β1·χ)/1 + exp (β0 + β1·χ), where Pr (death) is the probability of death, χ is each PK parameter (i.e., AUC, C_{max}, $T_{>IC95}$), and β0 and β1 are the parameters to be estimated. Model fitness for PK parameter in logistic regression model was evaluated by Akaike information criterion (AIC, Yamaoka et al., 1978). The Akaike Information Criterion (AIC) is widely applied to any set of maximum likelihood-based models including the logistic regression model for model-choice. Models with the smaller value of AIC are selected as the better models explaining the data.

3. Results

3.1. The susceptibility of various influenza viruses to NAIs

Initially, IC_{95} and IC_{50} values of peramivir trihydrate and oseltamivir carboxylic acid were measured for NA derived from various subtypes of influenza including 16 avian influenza viruses (Table 1). The IC_{95} values of peramivir trihydrate ranged between 4.64 and 22.42 nM (between 1.52 ng/ml and 7.36 ng/ml). These values were lower than those for oseltamivir carboxylic acid in almost all strains. The IC_{95} values against the A/WS/33 (H1N1) strain used for the antiviral study in mice were 5.94 nM (1.95 ng/ml) for peramivir trihydrate and 18.64 nM (5.30 ng/ml) for oseltamivir carboxylic acid.

3.2. PK evaluation of peramivir in infected mice

Next, peramivir plasma concentrations were measured in infected mice (Fig. 2). The concentration of peramivir increased dose proportionally from 0.4 to 10 mg/kg. Additionally, peramivir showed typical two-compartment model pharmacokinetics and rapid distribution and/or elimination in α (distribution) phase. Model parameters were calculated by using the average of plasma concentration in three doses and were as follows: Vc; 309.5 mL/kg, Ke; 3.002 h^{-1}, K_{12}; 0.313 h^{-1}, K_{21}; 0.204 h^{-1}. These values were used to calculate the indices of AUC, C_{max} and $T_{>IC95}$, for the investigation of the correlation to PD (Table 2).

3.3. Therapeutic efficacy of peramivir in infected mice

To investigate the relationship between PK parameter and *in vivo* antiviral activity, the measurement of PD outcome should be conducted in several dosing conditions which show various

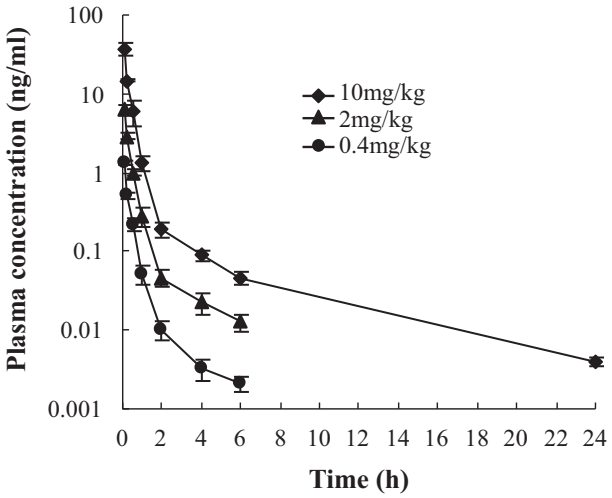

Fig. 2. Peramivir concentrations in mouse plasma following intravenous drug administration. Single treatments with peramivir were given at 48 h after the onset of an influenza A/WS/33 (H1N1) virus infection. Each symbol represents the mean plasma concentration of four mice in each point. Error bars correspond to 1 standard deviation.

Table 2
Dosing schedule and ranges of PK/PD parameter.

Number of doses	The range of PK/PD parameters[a]			
	Dose (mg/kg/shot)	AUC (ng·h/ml)	C_{max} (ng/ml)	$T_{>IC95}$ (h)
4 (q3 h)	0.0625–2	269–8611	203–6508	7.66–29.73
2 (q6 h)	0.125–4	269–8611	405–12,952	5.43–28.03
1 (q12 h)	0.25–8	269–8611	808–25,848	5.36–24.24

[a] The pharmacokinetic parameters at the each dosing regimen were estimated from the PK parameters obtained in single intravenous administration at 10 mg/kg, 2 mg/kg and 0.4 mg/kg by two-compartment model. Four mice were used in each group for the PK evaluation.

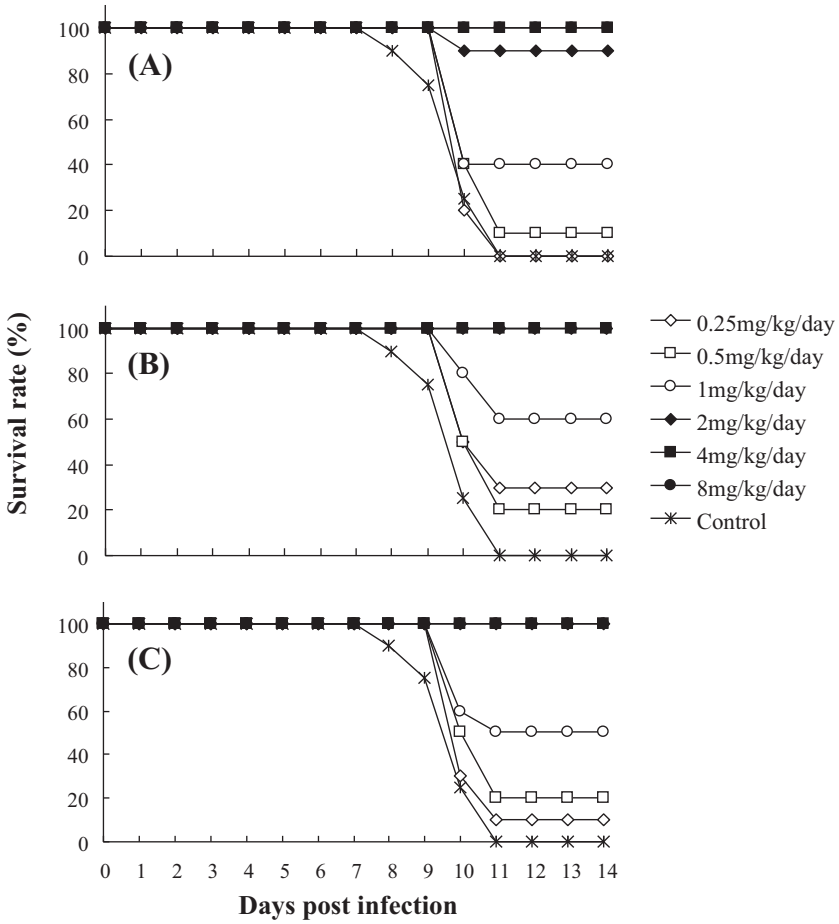

Fig. 3. Survival of mice infected with A/WS/33 (H1N1). A total of 18 types of dosing regimens (6 types of amount of dosages and 3 types of dose frequencies) were intravenously administrated starting 48 h after virus challenge. That is 0.25, 0.5, 1, 2, 4 and 8 mg/kg/day as total daily doses on three schedules: once (A), twice (every 6 h, B) and four times (every 3 h, C). Each symbol represents the survival rate of 10 mice in treatment groups or 20 mice in the control group.

combinations of three PK parameters. The therapeutic efficacy of peramivir in three dosing schedules and six daily dosage amounts against mice infected with influenza virus A/WS/33 (H1N1) was measured (Fig. 3). In the control group, fatalities were observed beginning at day 7 after infection and all mice were dead at day 11. The therapeutic efficacy was very similar in the three dosing schedules. For each schedule, survival improved with higher doses. Total daily doses of 4 mg/kg/day and 8 mg/kg/day of peramivir resulted in survival of all mice in all three dosing schedules. The data suggests not only exposure but also the dose is an important factor in treatment of influenza virus infection by peramivir.

3.4. PK/PD assessment of peramivir in infected mice

The relationship between each PK parameter, such as AUC, C_{max} and $T_{>IC95}$, and antiviral activity was estimated using a logistic regression model (Table 3, Fig. 4). The Wald test indicated that all three PK parameters have significant influence on antiviral activity ($p < 0.0001$). Moreover, the fitness for PK parameter in logistic regression model was evaluated by calculated AIC index (Table 3). AIC is a measure of the relative quality of a statistical

Table 3
The Estimated parameters and AIC value by logistic regression analysis.

PK/PD parameter		Estimated value	SE	p value[a]	AIC[b]
log (AUC)	β0	16.94	2.50	<0.0001	114
	β1	−2.50	0.36	<0.0001	
log (C_{max})	β0	11.53	1.74	<0.0001	151
	β1	−1.61	0.24	<0.0001	
$T_{>IC95}$	β0	5.14	0.81	<0.0001	124
	β1	−0.36	0.05	<0.0001	

[a] Determined by the Wald test.
[b] Akaike information criterion.

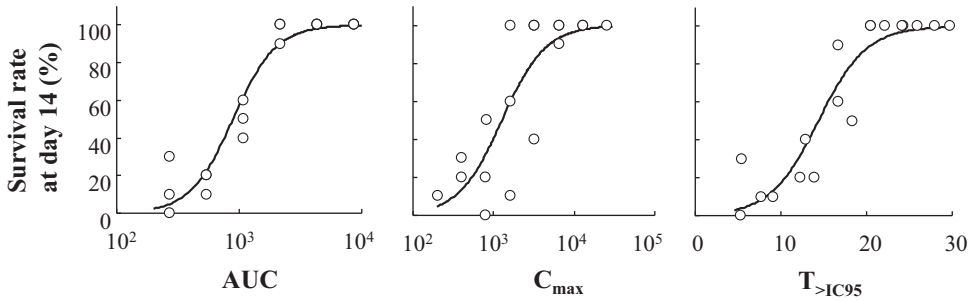

Fig. 4. The estimated logistic regression curve between each PK parameter and the survival rate of mice at day 14 after infected with influenza A/WS/33 (H1N1). Each symbol represent observed value and solid lines represent estimated curves.

model and smaller AIC index suggests that corresponding model is better fitting model (Yamaoka et al., 1978). The AIC indices of AUC, C_{max} and $T_{>IC95}$ were about 114, 151 and 124, respectively. The AIC indices of AUC and $T_{>IC95}$ were almost the same values and smaller than that of C_{max}. Therefore, both AUC and $T_{>IC95}$ were the PK parameters that correlated best with antiviral efficacy in mice infected with influenza virus. The AUC and $T_{>IC95}$ values for efficacy of 95% survival rate (maximal efficacy) were 2890 ng·h/ml and 23 h, respectively.

4. Discussion

Peramivir is expected to be of benefit for patients with influenza virus infection because it is the only intravenous formulation of an influenza antiviral and has shown potential for faster improvement in symptoms (Shetty and Peek, 2012; Shobugawa et al., 2012). To administer intravenous peramivir with maximal efficacy in the clinical setting, optimization of the dosage regimen based on a better understanding of PK/PD relationships would be helpful. The current study represents the first evaluation of the relationship between PK parameters and antiviral activity of intravenous peramivir in a mouse model of influenza A/H1N1 infection.

This study demonstrated that both AUC and $T_{>IC95}$ were the pharmacodynamically responsive PK parameters for *in vivo* antiviral activity of intravenous peramivir. Other report regarding PK/PD analysis of orally administered peramivir indicated that AUC was the most responsive PK parameter for efficacy in a mouse model of A/H3N2 infection, in this case by fitting to a Cox proportional hazards model (Drusano et al., 2001). The result in this study and other report suggested that AUC would be the pharmacodynamically responsive PK parameter for peramivir, regardless of influenza virus type or administration route. In a Phase III study, the reduction of viral titer observed with peramivir 300 mg, twice a day and peramivir 600 mg, once a day in patients hospitalized with confirmed or suspected 2009 pandemic influenza A/H1N1 were similar (Shetty and Peek, 2012). That result also suggested that the amount of exposure, i.e., AUC, was associated with efficacy. Moreover, the PK/PD relationships of other neuraminidase inhibitors, such as oseltamivir or zanamivir, investigated in an *in vitro* hollow fiber infection model (McSharry et al., 2009; Brown et al., 2011a, 2011b) also suggested that AUC and $T_{>EC50}$ were the parameters which correlated with efficacy. Therefore, AUC seems to be a consistent PD-responsive PK parameter for efficacy among all neuraminidase inhibitors. The IC_{95} value was adopted for this PK/PD analysis because the IC_{50} of peramivir trihydrate against A/WS/33 is 0.10 ng/ml (0.31 nM), which is much lower than the detection limit of quantification in PK study. More experiments will be necessary to lead to the definitive conclusion about the correlation between time-dependent PK parameter ($T_{>IC95}$, $T_{>IC50}$, $T_{>EC50}$, etc.) and antiviral efficacy.

PK/PD analysis of orally administrated peramivir for experimental virus infection in healthy volunteers was conducted and AUC was the PK parameter which correlated with the reduction of viral titer (Iyer et al., 2002) and the AUC values for 50% efficacy was 1089 ng·h/mL for influenza A. Furthermore, PK/PD relationship for efficacy of oseltamivir in clinical study demonstrated that lowering composite symptom score and shortening time to alleviation of composite symptoms was confirmed under the condition in AUC value of >1495 ng·h/mL and >1568 ng·h/mL, respectively (Rayner et al., 2013). This study showed that AUC for 50% and 90% survival rate was 889 ng·h/mL and 2890 ng·h/mL which was very close to clinical studies described above. The result in this study was well reflected on the clinical setting of peramivir. Furthermore, this PK/PD model was thought to be the supportive evidence for the efficacy of all NAIs, regardless administration route, in clinical setting.

In this study, the indices of AUC and $T_{>IC95}$ for 95% efficacy were 2890 ng·h/ml and 23 h, respectively. In a clinical study in influenza patients, the AUC after intravenous peramivir 300 mg was about 40,000 ng·h/ml (Sugaya et al., 2012) which is more than 10-fold greater than the value calculated for maximal efficacy (efficacy for 95% survival rate) in this mouse study. With 300 mg as a single dose, the median plasma concentration of peramivir from 18 to 24 h after the end of infusion was 17.4–20.7 ng/ml (Kohno et al., 2011b; Sugaya et al., 2012) and the $T_{>IC95}$ are expected to far exceed 24 h. The *in vitro* susceptibility range of peramivir trihydrate for clinical isolates is comparable to that of A/WS/33 (IC_{50}: 0.31 nM) used in this study (Feng et al., 2012). In consideration of these data, the therapeutic dose of peramivir in clinical use would appear to be adequate, and indeed good outcomes were confirmed in several clinical studies (Kohno et al., 2011a, 2011b; Sugaya et al., 2012).

In March 2013, the National Health and Family Planning Commission of China announced that three human infections with novel influenza virus were confirmed in Shanghai and these viruses were identified as novel reassortant avian influenza virus A (H7N9) (Rongbao et al., 2013; Liu et al., 2013). These patients showed rapidly progressive lower respiratory tract infection which caused death several days after the onset illness (Rongbao et al., 2013). This outbreak of novel avian influenza has raised serious concern regarding its potential for pandemic spread. In this study, the IC_{95} values of peramivir trihydrate for 16 avian influenza viruses ranged between 1.52 ng/ml and 7.36 ng/ml (between 4.64 ng/ml and 22.4 ng/ml), and for two H11N9 subtypes were 2.43 ng/ml and 2.81 ng/ml (7.40 nM and 8.56 nM) respectively, which are comparable to that against A/WS/33 (H1N1) (Table 1). The susceptibility of H7N9 subtype (A/Shanghai/1/13) isolated from China in 2013 to peramivir reported with the IC_{50} value of 0.20 ng/ml (0.6 nM, Yen et al., 2013) which is also comparable to that for A/WS/33 (H1N1) and the indices of AUC or $T_{>IC95}$ needed for maximal efficacy would be exceeded by standard clinical doses of peramivir, based on the PK/PD considerations in this study. This suggests that intravenous peramivir should be considered as a therapeutic option for patients with infection of novel avian influenza viruses.

Non-clinical PK/PD studies for the other NAIs did not calculate the indices needed for sufficient efficacy, and it is unclear whether the indices differ substantially among the four available NAIs. Further study will be needed to address this question. The survival rate at day 14 was defined as the antiviral activity outcome measure for this study. Though survival rate is the principal end point to evaluate antiviral activity in murine infection models, reduction of viral titer in the lung is an important additional end point and should also be evaluated as an antiviral activity outcome measure for *in vivo* PK/PD studies.

This study found correlations between peramivir AUC and $T_{>IC95}$ and antiviral activity and related these findings to clinical efficacy of intravenous peramivir 300 mg. However, relating animal PK/PD findings to the clinic is challenging, because of difference in clearance of antiviral agents, which is also spices specific, differences in endpoints or time course, and the absence of simple and standard PD-responsive PK parameters, such as minimum inhibitory concentration in antimicrobials (Schmidt et al., 2008).

Acknowledgements

All work reported here was financially supported by Shionogi & Co., Ltd. The authors except for H.K. and Y.S. are all employees of Shionogi Co., Ltd.

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