

# Neuraminidase sequence analysis and susceptibility to peramivir of influenza viruses isolated from clinical trials

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## Abstract

### Background:

Peramivir (Rapiacta®) is an influenza neuraminidase (NA) inhibitor approved for sale in January 2010 in Japan. In this study, we investigated the change in inhibitory effect on NA activity before and after treatment with peramivir and how the change in the inhibitory effect correlated with the clinical efficacy.

### Methods:

Against clinical isolates from four peramivir clinical studies in adult and pediatric patients during 2007-2010 Japanese influenza epidemic, the inhibitory effect of peramivir was measured by NA inhibition assay. Measurement of Inhibitory effect of other NA inhibitors and sequence analysis of NA gene were performed when the isolates showed reduced susceptibility (>3 fold) to peramivir. Deduced amino acid was compared with that from sensitive isolates and mutations responsible for reduced susceptibility were predicted.

### Results:

Out of 1074 patients treated with peramivir, post-treatment influenza viruses with reduced susceptibility to peramivir were found in 13 patients (1.2%, 13/1074): 2.6% (5/196, adults) for 2007-2008, 0.1% (1/763, adult) for 2008-2009 and 6.1% (7/115, children) for 2009-2010. Almost all these viruses carried A/H1N1 with H275Y mutation in NA and exhibited 10-50-fold reduction in susceptibility to peramivir compared to the isolates at baseline. The double mutation of V94I and R152K in 2009 A/H1N1 viruses was also detected in an isolate from a pediatric patient, causing a 10-fold reduction in susceptibility to peramivir compared to the isolate at baseline. In the 13 patients, the median time to alleviation of symptoms was 45.0 hours (95% CI: 20.4-70.9) compared to 68.5 hours (95% CI: 64.3-75.7) in the remaining 1061 patients with no viruses that showed reduction in susceptibility to peramivir.

### Conclusions:

In 1074 patients treated with peramivir, low NA susceptibility to peramivir with amino acid mutation was detected in 13 patients after treatment. However, clinical worsening was not observed in any of these patients.

## Introduction

For influenza virus infection, to examine whether resistant virus emerges in the course of treatment of NA inhibitor is important to provide appropriate therapeutic strategies. Sometimes isolated viruses for the patients have mutated NA with lower susceptibility to the inhibitor in *in vitro* enzymatic assay compared to pre-treatment NA. However, how the decreased NA susceptibility to the inhibitor affects response to treatment and clinical efficacy is still unclear. In this study, we investigated the change in NA susceptibility to peramivir before and after treatment with peramivir and how the change in the inhibitory effect correlated with the clinical efficacy.

## Materials and Methods

### Study patients:

1074 patients with influenza virus infection were treated peramivir in four clinical studies of peramivir conducted in Japan (Adult Phase I 196 patients in 2007-08<sup>1</sup>, Adult Phase III single dosing 726 patients in 2008-09<sup>2</sup>, Adult Phase III high-risk 37 patients in 2008-09<sup>3</sup>, Children Phase III 115 patients in 2009-10<sup>4</sup>).

### Collection of samples:

A nasal swab from one nostril and a single throat swab were collected at days 1 (baseline) and at subsequent time points. These samples were divided for typing and gene sequencing using PCR, virus titration and NA enzyme inhibitory assay.

### Virus titration:

Titers were determined by the 50% Tissue Culture Infectious Dose (TCID<sub>50</sub>) in Madin-Darby canine kidney (MDCK) cells.

### NA enzyme inhibition assay (NIA):

Virus, peramivir and substrate (2-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA)) were mixed. After 30 min reaction, the fluorometric intensity of 4-methylumbelliferon released from MUNANA was measured. The 50% inhibitory concentration (IC<sub>50</sub>) was calculated by plotting the percent inhibition of NA activity versus peramivir concentration.

### NA gene sequencing:

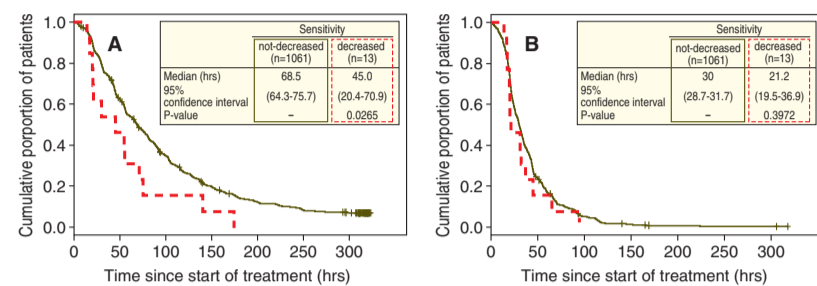
cDNA was generated from viral RNA. The DNA fragment of whole or a portion of the NA region was amplified from the cDNA with TaKaRa Ex Taq and PCR primers. The PCR products were purified, sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions (Applied Biosystems), and analyzed on a DNA sequencer. Amino acid substitutions of the NA region were investigated.

**Table 1. IC<sub>50</sub> change of peramivir against NA and responsible amino acid mutation of influenza viruses isolated from patients**

Study (season)	Patient ID	Gender	Age (years)	Influenza subtype	IC <sub>50</sub> in NIA (nM)		Fold reduction in IC <sub>50</sub>	Amino acid mutation in NA
					before treatment (day1)	after treatment (day2-6)		
Ph II (2007-08)	080-1	Female	38	A/H1	0.966	14.5	15	H275Y
	086-5	Female	39	A/H1	1.72	31.5	18	H275Y
	135-6	Female	21	A/H1	1.35	27.6	20	H275Y
	148-4	Male	22	A/H1	1.12	27.8	25	H275Y
	163-5	Female	25	A/H1	1.47	30.3	21	H275Y
Ph III (2008-09)	719-3	Male	20	A/H1 and/or H3	0.835 (H3)	21.5 (H1)	26	H275Y
Pediatric (2009-10)	3FA010	Female	0.5	A/H1pdm	0.378	20.2	53	H275Y
	3AH001	Female	1	A/H1pdm	0.349	19.1	55	H275Y
	3BC004	Female	11	A/H1pdm	0.468	24.6	52	H275Y
	3BG005	Female	4	A/H1pdm	0.343	3.48	10	V94I, R152K
	3BH008	Female	11	A/H1pdm	0.326	17.4	53	H275Y
	3BP007	Female	15	A/H1pdm	0.363	19.1	53	H275Y
	3BR002	Female	5	A/H1pdm	0.302	16.8	55	H275Y

- No isolates from Ph III high-risk study patients showed 3-fold reduction in IC<sub>50</sub> before and after peramivir's treatment.
- Almost all viruses with reduced NA susceptibility (15-55-fold reduction in IC<sub>50</sub>) carried the H275Y mutation in NA/H1N1.

**Figure 1. Time to alleviation of symptoms (A) and resolution of fever (B) in clinical studies of peramivir (three studies combined)**



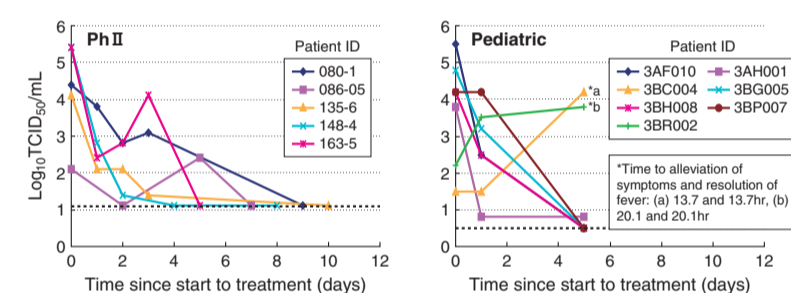
**Table 2. Time to alleviation of symptoms and resolution of fever (individual studies\*)**

	Ph II study		Pediatric study		
	Sensitivity		Sensitivity		
	not-decreased (n=191)	decreased (n=5)	not-decreased (n=108)	decreased (n=7)	
Symptoms	Median (hrs)	59.6	54.4	28.1	20.4
	95% confidence interval	54.5-66.8	29.9-174.1	22.0-31.8	17.0-70.9
	P-value		0.8279		0.2589
Fever	Median (hrs)	32.4	29.5	20.5	20.1
	95% confidence interval	19.5-94.8	27.3-31.5	18.9-20.9	16.5-21.2
	P-value		0.2743		0.5605

\*Ph III data was not shown because the mutated virus (H275Y) was not clear whether it existed before treatment.

- Clinical efficacy of peramivir was not significantly different between patients in whom susceptibility to peramivir was decreased and not-decreased in response to treatment.

**Figure 2. Change in influenza virus titer (individual studies)**



- Even though susceptibility to peramivir was decreased after treatment, viral titer decreased to undetectable level (dotted line) around 7 days in Ph II and 5 days in pediatric study.

**Table 3. H275Y and additional 2 amino acid mutations associated with viral fitness\*5**

Study	Season	subtype	isolation point	ratio	Amino acid of NA region		
					R222Q	V234M	H275Y
Ph II	2007-2008	H1N1 (n=65)	Before dosing	6/65 (9.2%)	Q	M	H
				1/65 (1.5%)	Q	M	Y
				58/65 (89%)	N.T.	N.T.	N.T.
			After dosing	1/65 (1.5%)	Q	M	H
				7/65 (11%)	Q	M	Y
				57/65 (88%)	N.T.	N.T.	N.T.
Ph III (single-dose)	2008-2009	H1N1 (n=321)	Before dosing	1/321 (0.3%)	Q	M	H
			After dosing	320/321 (99.8%)	Q	M	Y
			321/321 (100%)	N.T.	N.T.	N.T.	
Pediatric	2009-2010	H1N1pdm (n=106)	Before dosing	106/106 (100%)	Corresponding amino acids in A/H1N1pdm are not clear		
			After dosing	6/106 (5.1%)			Y

N.T.: Not tested

- It was reported H275Y attenuates seasonal H1N1 unless there are permissive secondary mutations (R222Q and V234M)\*5. These mutations were detected in our study from 2007 to 2009\*5

## Conclusions

- In 1074 patients treated with peramivir, isolates with reduced susceptibility (>3 fold) to peramivir were detected in 13 patients after treatment (1.2%, IC<sub>50</sub> range: 20 -50 nM). All 13 isolates were A/H1N1. Twelve had H275Y; the other had V94I and R152K mutation in NA.
- However, these patients did not show a tendency to prolonged time to alleviation of symptoms or resolution of fever. Clinical worsening was not clearly observed in any of these patients.
- Regarding A/H1N1pdm with H275Y, secondary mutations to recover viral fitness have not been reported, so far. Further analysis on whether there is correlation between additional mutation and change of viral fitness might help the probability of H275Y epidemic.

## Reference

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