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Influenza Polymerase Inhibitors: Mechanisms of Action and Resistance

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The influenza virus RNA-dependent RNA polymerase is highly conserved among influenza A, B, C, and D viruses. It comprises three subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) in influenza A and B viruses or polymerase 3 protein (P3) in influenza C and D viruses. Because this polymerase is essential for influenza virus replication, it has been considered as a target for antiviral agents. Recently, several polymerase inhibitors that target each subunit have been developed. This review discusses the mechanism of action, antiviral activity, and emergence of resistance to three inhibitors approved for the treatment of influenza or in late-phase clinical trials: the PB1 inhibitor favipiravir, the PB2 inhibitor pimodivir, and the PA inhibitor baloxavir marboxil.

The influenza virus RNA-dependent RNA polymerase is highly conserved among influenza A, B, C, and D viruses (Peng et al. 2019). It consists of three subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) in influenza A and B viruses or polymerase 3 protein (P3) in influenza C and D viruses (Reich et al. 2014; Hengrung et al. 2015; Fan et al. 2019; Peng et al. 2019).

The RNA-dependent RNA polymerase is essential for the viral replication cycle, including transcription and viral protein synthesis, as well as viral genome replication (Stevaert and Nae-sens 2016). Primary transcription of the viral genome RNA (vRNA) to mRNA starts with the cap-snatching reaction, in which the 5'-terminal 7-methylguanosine (m⁷G) cap structure of the host mRNA is bound by the cap-binding

domain of PB2 and cleaved by the cap-dependent endonuclease of PA at 10–13 nucleotides from the cap, to yield primers for viral mRNA synthesis. Replication of vRNA then proceeds to yield the full-length complementary RNA (cRNA). During both transcription and replication, RNA elongation is carried out by PB1.

The polymerase active sites for RNA binding, cleavage, and elongation are conserved among the various types of influenza viruses, which is why the RNA-dependent RNA polymerase has been considered as a target for antiviral agents. Recently, several polymerase inhibitors that target each subunit have been developed. This review provides an overview of three such inhibitors approved for the treatment of influenza or in late-phase clinical trials: the PB1 inhibitor favipiravir, the PB2 inhibitor pimodivir, and the PA inhibitor baloxavir marboxil (Table 1).

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Table 1. Overview of influenza polymerase inhibitors

	Favipiravir	Pimodivir	Baloxavir marboxil
Alternative name	T-705	JNJ-63623872, VX-787	S-033188
Molecular formula	C ₅ H ₄ FN ₃ O ₂	C ₂₀ H ₁₉ F ₂ N ₅ O ₂	C ₂₇ H ₂₃ F ₂ N ₃ O ₇ S
Molecular weight	157.1	399.4	571.6
Active form	Favipiravir-RTP	–	Baloxavir acid
Target protein	PB1	PB2	PA
Target influenza viruses	Types A, B, C, D	Type A	Types A, B, C, D
Current status	Approved in Japan for influenza pandemic preparedness	In Phase III clinical trials	Approved in several countries (Japan, USA, etc.)
Route of administration	BID oral dose for 5 d	BID oral dose for 5 d	Single oral dose
Inhibition of M2 inhibitor-resistant viruses	Yes	Yes	Yes
Inhibition of NA inhibitor-resistant viruses	Yes	Yes	Yes
Emergence of variants with reduced susceptibility in vitro	Yes	Yes	Yes
Emergence of variants with reduced susceptibility in clinical trials	No	Yes	Yes

(Favipiravir-RTP) Favipiravir-ribofuranosyl-5'-triphosphate, (PB1) polymerase basic protein 1, (PB2) polymerase basic protein 2, (PA) polymerase acidic protein, (BID) twice daily, (M2) matrix protein 2, (NA) neuraminidase.

FAVIPIRAVIR

Favipiravir (T-705) was developed in Japan and approved for influenza pandemic preparedness in Japan on March 24, 2014 (<http://www.pmda.go.jp/files/000210319.pdf>). Because favipiravir increases the risk for teratogenicity and embryotoxicity, conditional marketing approval with strict regulations for its production and clinical use was granted (Furuta et al. 2017). Accordingly, favipiravir is available only for patients with novel or reemerging pandemic influenza virus infection in whom other influenza antivirals are ineffective or not sufficiently effective. In other countries, favipiravir is under investigation. Two multinational phase 3 clinical trials of favipiravir in adults with uncomplicated influenza were completed in 2015 (NCT02008344; NCT02026349). A phase 2a clinical trial of favipiravir in hospitalized patients aged 18 yr and older with severe influenza was completed in China in March 2019 (NCT03394209).

Mechanism of Action

Favipiravir, a pyrazine derivative, was discovered through the screening of a chemical library

for compounds with in vitro anti-influenza virus activity (Furuta et al. 2002). Favipiravir undergoes phosphoribosylation and further phosphorylation in infected cells to achieve its active form, favipiravir-ribofuranosyl-5'-triphosphate (favipiravir-RTP) (Furuta et al. 2005).

Favipiravir acts as a chain terminator or a mutagen (Vanderlinden et al. 2016; Goldhill et al. 2019). It is recognized as a purine analog by the RNA-dependent RNA polymerase and efficiently incorporated into a nascent RNA strand as a guanosine and an adenosine analog (Jin et al. 2013; Sangawa et al. 2013). This prevents the incorporation of GTP and ATP in a competitive manner and inhibits RNA elongation, which is carried out by PB1. Furthermore, favipiravir causes lethal mutagenesis by increasing the G-to-A and C-to-U mutation frequency and generating nonviable progeny virus (Baranovich et al. 2013).

Antiviral Activity

Favipiravir has activity against the RNA-dependent RNA polymerase of influenza and other RNA viruses but not human DNA-dependent

RNA and DNA polymerases (Furuta et al. 2013). Favipiravir inhibits the replication of influenza A, B, C, and D viruses, including matrix protein 2 (M2) inhibitor-resistant viruses and neuraminidase (NA) inhibitor-resistant viruses (Furuta et al. 2002; Mishin et al. 2019).

Synergistic effects of favipiravir and an NA inhibitor (oseltamivir, peramivir, zanamivir, or laninamivir) have been demonstrated in vitro (Tarbet et al. 2014) and in mice (Smee et al. 2010, 2013; Tarbet et al. 2012; Park et al. 2014; Marathe et al. 2016; Baz et al. 2018; Kiso et al. 2018). A phase 2a clinical trial of favipiravir suggested that combination therapy of favipiravir and oseltamivir may accelerate clinical recovery in hospitalized patients aged 18 yr and older with severe influenza (Wang et al. 2019).

Resistance

Ten in vitro passages of A/England/195/2009 (H1N1)pdm09 virus in the presence of favipiravir led to the acquisition of a K229R substitution in PB1 that conferred a 31-fold reduction in susceptibility to favipiravir (Goldhill et al. 2018). The PB1 K229R substitution prevented the incorporation of favipiravir into nascent viral RNA and decreased the mutation frequency in the presence of favipiravir (Goldhill et al. 2018). Although the PB1 K229R substitution significantly reduced the viral polymerase activity and replication fitness, a P653L substitution in PA compensated for the fitness costs. The PB1 K229R substitution is conserved among different influenza subtypes and confers reduced favipiravir susceptibility to the RNA-dependent RNA polymerase of A/Victoria/3/1975(H3N2) and A/Anhui/1/2013(H7N9) viruses (Goldhill et al. 2018).

In a mouse model infected with the highly pathogenic A/Turkey/15/2006(H5N1) virus, emergence of resistance was not observed in both the favipiravir monotherapy and favipiravir plus oseltamivir combination therapy groups (Marathe et al. 2016). In immunodeficient mice infected with A/Quebec/144147/2009(H1N1)pdm09 virus (Baz et al. 2018) and immunocompromised nude mice infected with A/California/04/2009(H1N1)pdm09 virus (Kiso et al. 2018),

combination therapy with favipiravir and NA inhibitor, oseltamivir, or laninamivir did not induce variants with reduced susceptibility to favipiravir, although variants with reduced susceptibility to the NA inhibitors did emerge.

In phase 3 clinical trials of favipiravir, some amino acid substitutions in PB1, PB2, and/or PA emerged after favipiravir treatment (Takashita et al. 2016). A L666F substitution in PA conferred reduced polymerase activity to A(H1N1)pdm09 virus, although no variants with reduced susceptibility to favipiravir emerged during the clinical trials.

PIMODIVIR

Pimodivir (JNJ-63623872, VX-787) received Fast Track designation by the Food and Drug Administration (FDA) in March 2017 because of its potential to address an unmet medical need in those who develop influenza A infection and are hospitalized or at high risk of related complications (Finberg et al. 2019). Two phase 3 clinical trials of pimodivir are ongoing in hospitalized patients and high-risk outpatients aged 13–85 yr with influenza A infection (NCT03376321; NCT03381196). In both clinical trials, the efficacy and safety of pimodivir in combination with the standard-of-care treatment, which may include influenza antivirals and/or supportive care only, are being evaluated.

Mechanism of Action

Pimodivir is a cyclohexyl carboxylic acid analog obtained by screening a compound library for compounds with in vitro activity against influenza virus (Clark et al. 2014). In the cap-snatching reaction, PB2 binds to the m⁷G cap structure of host mRNA (Stevaert and Naesens 2016). Pimodivir occupies the cap-binding domain of PB2 and inhibits RNA binding (Clark et al. 2014; Byrn et al. 2015; Stevaert and Naesens 2016). Pimodivir forms hydrogen bonds with E361 and K376 in the PB2 cap-binding domain. The azaindole ring of pimodivir is sandwiched between H357 and F404, whereas the pyrimidine ring interacts with F323. The carboxylic acid of pimodivir makes water-mediated inter-

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actions with R355, H357, and Q406 (Clark et al. 2014; Byrn et al. 2015).

Antiviral Activity

Pimodivir has activity against influenza A viruses, including M2 inhibitor-resistant viruses and NA inhibitor-resistant viruses (Clark et al. 2014; Byrn et al. 2015), but showed negligible activity against influenza B viruses (Byrn et al. 2015). Several key amino acids in the PB2 cap-binding domain differ between influenza A and B viruses (Liu et al. 2015), and some residues in the cap-binding domain of influenza B virus could sterically hinder pimodivir (Severin et al. 2016). Furthermore, the cap-binding affinity and specificity of influenza B virus differ from those of influenza A virus (Liu et al. 2015): influenza B virus has weaker cap-binding affinity than influenza A virus (Wakai et al. 2011); and influenza A virus has strict specificity for the m⁷G cap structure, whereas influenza B virus recognizes the unmethylated cap structure (Wakai et al. 2011).

Synergistic effects have been demonstrated in vitro for pimodivir and favipiravir, oseltamivir, or zanamivir (Byrn et al. 2015). In the phase 2b TOPAZ clinical trial of pimodivir (NCT02342249), combination therapy of pimodivir plus oseltamivir resulted in a significantly lower viral load compared with pimodivir monotherapy in adults with acute uncomplicated influenza A (Finberg et al. 2019). The phase 2b OPAL clinical trial of pimodivir (NCT02532283), which compared the combination therapy of pimodivir plus oseltamivir to oseltamivir monotherapy, showed shorter durations of virus detection and illness for the combination therapy compared with oseltamivir monotherapy in hospitalized patients aged 18–85 yr enrolled within 72 h of symptom onset (Hayden and Shindo 2019; Mifsud et al. 2019).

Resistance

In vitro passages of A/Puerto Rico/8/34(H1N1) virus in the presence of pimodivir identified six amino acid substitutions in PB2 (i.e., Q306H, S324I, S324N, S324R, F404Y, and N510T) that were associated with reduced susceptibility to

pimodivir (Byrn et al. 2015). These substitutions conferred 63- to 257-fold reduced susceptibility to pimodivir and are rare in naturally occurring human influenza viruses (0%–0.03%), suggesting that they are treatment-emergent substitutions.

In a mouse model of A/Puerto Rico/8/34 (H1N1) virus infection, emergence of resistance was not observed (Byrn et al. 2015).

In a phase 2a clinical trial of pimodivir, potential pimodivir-resistant variants with amino acid substitutions in PB2 (i.e., S324C, K376R, M431L/R/V, and M431I) were identified between days 3 and 7 of treatment in 9.7% (7/72) of pimodivir-treated volunteers infected with A/Wisconsin/67/2005(H3N2) virus (Trevejo et al. 2018). These volunteers had both high viral titers and high mean steady state minimum pimodivir concentrations in the plasma. The PB2 M431I substitution was detected in four of seven volunteers and conferred 57-fold reduced susceptibility to pimodivir, which was associated with a 13-fold reduction in replication capacity compared with the wild-type virus. Emergence of these substitutions was not coincident with viral rebound and all subjects subsequently cleared the virus after treatment for 5 d once daily.

In the phase 2b TOPAZ clinical trial of pimodivir, A(H1N1)pdm09 or A(H3N2) viruses with PB2 substitutions or reduced susceptibility to pimodivir were detected in 6.9% (4/58), 10.5% (6/57), and 1.8% (1/57) of patients treated with pimodivir 300 mg, pimodivir 600 mg, and a combination of pimodivir 600 mg and oseltamivir, respectively (Finberg et al. 2019). The PB2 substitutions detected in these patients (i.e., S324K/N/R, F325L, S337P, K376N/R, T378S, and N510K) were associated with 9.4- to >372-fold reduced susceptibility to pimodivir.

BALOXAVIR MARBOXIL

Baloxavir marboxil (S-033188) was developed in Japan and approved there on 23 February 2018 for the treatment of influenza A and B virus infection in patients aged 12 yr and older and children younger than 12 yr weighing ≥10 kg (<http://www.pmda.go.jp/files/000225380.pdf>).

Table 2. Influenza antiviral supply to medical institutes in Japan

Influenza season	Doses (×10,000)				
	NA inhibitors				Polymerase inhibitor
	Zanamivir	Oseltamivir	Peramivir	Laninamivir	Baloxavir
2009–2010	515	859	0.5	–	–
2010–2011	54	240	13	166	–
2011–2012	127	356	26	270	–
2012–2013	110	358	35	280	–
2013–2014	151	336	37	330	–
2014–2015	132	422	37	400	–
2015–2016	40	375	40	368	–
2016–2017	151	453	50	485	–
2017–2018	158	570	60	690	40
2018–2019	59	464	32	289	528

Data adapted from notification on stable supply of influenza antivirals from the Ministry of Health, Labour, and Welfare, Japan.

(NA) Neuraminidase, (–) unapproved.

The FDA granted a Priority Review to baloxavir, under which the FDA takes expedited action on an application to determine whether the drug, if approved, would significantly improve the safety or effectiveness of treating, diagnosing, or preventing a serious condition. Indeed, baloxavir was approved in the United States on 24 October 2018 (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210854Orig1s000TOC.cfm) and in several other countries in 2019 for patients aged 12 yr and older weighing at least 40 kg. Because it is not clear whether the selected doses of 40 mg or 80 mg for subjects weighing ≥ 80 kg are adequate to control influenza B virus infections, the FDA has recommended that higher doses of baloxavir be evaluated (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210854Orig1s000MicroR.pdf). The phase 3 MINISTONE-2 clinical trial of baloxavir in patients 1 to <12 yr of age was completed in the United States and other countries in April 2019 (NCT03629184). In this trial, higher doses of baloxavir (2 mg/kg for subjects weighing <20 kg and 40 mg for ≥ 20 kg), which were 2- to 4-fold higher than those approved in Japan (10 mg for subjects weighing 10 to <20 kg, 20 mg for 20 to <40 kg, and 40 mg for ≥ 40 kg), were administered.

In the 2018–2019 influenza season, baloxavir was supplied to medical institutions that to-

gether served about 5.3 million people in Japan (i.e., one-twenty-fourth of the Japanese population) and became the most used influenza antiviral in Japan (Table 2).

Mechanism of Action

Baloxavir acid (S-033447), an active form of baloxavir marboxil, was generated by rational molecular design based on the two-metal pharmacophore concept for dolutegravir, an inhibitor of human immunodeficiency virus (HIV) integrase, because influenza PA and HIV integrase both use two divalent metal ions as cofactors for their endonuclease activity (Omoto et al. 2018). To enhance oral absorption of baloxavir acid, a phenolic hydroxyl group was added, thereby creating baloxavir marboxil (Omoto et al. 2018).

Baloxavir marboxil is rapidly hydrolyzed by esterases in the intestine, liver, and blood into baloxavir acid (Koshimichi et al. 2019). Baloxavir acid binds to the PA endonuclease domain and inhibits RNA cleavage by the PA cap-dependent endonuclease (Omoto et al. 2018). Baloxavir acid forms van der Waals interactions with A20, Y24, K34, A37, and I38 in influenza A virus or with T20, F24, M34, N37, and I38 in influenza B virus in the PA endonuclease domain (Omoto et al. 2018).

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Antiviral Activity

Baloxavir has activity against influenza A, B, C, and D viruses, including NA inhibitor-resistant viruses (Noshi et al. 2018; Mishin et al. 2019). Influenza B viruses are less susceptible to baloxavir than are influenza A viruses (Takashita et al. 2018; Gubareva et al. 2019; Koszalka et al. 2019); the susceptibility pattern is influenza A>B>C>D (Mishin et al. 2019).

Synergistic effects of baloxavir and the NA inhibitor oseltamivir have been reported in vitro and in mice (Fukao et al. 2019). A phase 3 clinical trial of baloxavir to evaluate its efficacy and safety in combination with a standard-of-care NA inhibitor (oseltamivir, peramivir, or zanamivir) in hospitalized patients aged 12 yr and older with severe influenza is currently recruiting patients (NCT03684044).

Resistance

Six to nine in vitro passages of A/WSN/33 (H1N1) virus in the presence of baloxavir revealed that an I38T substitution in PA conferred 22- to 41-fold reduced susceptibility to baloxavir (Noshi et al. 2018). The PA I38T substitution occurs infrequently in naturally occurring A(H1N1)pdm09, A(H3N2), and B viruses (0%–0.02%) (Stevaert et al. 2013; Takashita et al. 2019b), suggesting that this substitution emerges as a result of treatment.

In a mouse model of highly pathogenic A/Guangdong/17SF003/2016(H7N9) virus infection, emergence of resistance has not been observed (Kiso et al. 2019a). In contrast, baloxavir induced the PA I38M and E199G substitutions associated with reduced susceptibility to baloxavir (Gubareva et al. 2019) in immunocompromised nude mice infected with A/California/04/2009(H1N1)pdm09 virus (Kiso et al. 2019b), although variants with reduced susceptibility to baloxavir did not emerge in immunosuppressed mice infected with A/Puerto Rico/8/34(H1N1) virus (<http://www.pmda.go.jp/files/000225380.pdf>).

In phase 2 and 3 clinical trials of baloxavir, the PA E23K, I38T, and I38F substitutions in A(H1N1)pdm09 viruses and the PA E23G/K,

A37T, I38T, I38M, and E199G substitutions in A(H3N2) viruses emerged after baloxavir treatment (Hayden et al. 2018; Omoto et al. 2018; Hirotsu et al. 2019). These substitutions conferred 4.5- to 57-fold reduced susceptibility to baloxavir (Omoto et al. 2018). The PA I38 substitution was the most frequently detected mutation and had a greater impact on baloxavir susceptibility than did the other substitutions. Most of the PA I38 substitutions (52.2%) were detected in children aged <6 yr infected with A(H3N2) virus (Table 3). Patients infected with PA I38 mutant viruses exhibited prolonged virus shedding, rebound in virus titers, and delayed symptom alleviation (Hayden et al. 2018; Hirotsu et al. 2019; Uehara et al. 2019).

In Japan, nationwide monitoring of the baloxavir susceptibility of influenza viruses was initiated in the 2017–2018 influenza season (Takashita et al. 2018). During this monitoring, clinical specimens are randomly collected from patients regardless of whether or not they were treated with NA inhibitors or baloxavir (<https://www.niid.go.jp/niid/en/influ-resist-e.html>). During the 2018–2019 season, 2.3% (9/395) of A(H1N1)pdm09 and 8.0% (34/424) of A(H3N2) viruses were found to possess the PA I38 substitutions (Table 4). Eight of these PA I38 mutant viruses were isolated from patients without prior baloxavir exposure, indicating human-to-human transmission of these mutant viruses (Takashita et al. 2019a,c).

CONCLUDING REMARKS

Previous studies using laboratory strains demonstrated that A/WSN/33(H1N1) and A/Victoria/3/75(H3N2) viruses with the PA I38T substitution showed decreased susceptibility to baloxavir and reduced replication fitness in vitro (Noshi et al. 2018; Omoto et al. 2018). In contrast, Checkmahomed et al. (2020) reported that the PA I38T substitution did not alter the replication fitness of the contemporary influenza viruses A/Quebec/144147/2009(H1N1)pdm09 and A/Switzerland/9715293/2013(H3N2) in vitro or in mice. Furthermore, A(H1N1)pdm09 and A(H3N2) viruses with the PA I38T substitution isolated from baloxavir-treated patients

Table 3. Influenza viruses with I38 substitutions in PA detected during baloxavir clinical trials

Clinical trial	Age group (years)	Type/subtype		
		A(H1N1)pdm09	A(H3N2)	B
Phase 3 in Japan	<6	20.0% (1/5)	52.2% (12/23)	0% (0/13)
Tablet: T0822	6 to <12	0% (0/2)	18.9% (10/53)	0% (0/3)
Granule: T0833				
Phase 2: T0821	≥12	3.4% (4/116)	10.3% (35/341)	0% (0/87)
Phase 3: T0831				
CAPSTONE-1 (NCT02954354)				
Phase 3: T0832	12 to <65	7.1% (1/14)	7.1% (7/99)	1.1% (1/88)
CAPSTONE-2 (NCT02949011)	≥65	0% (0/3)	14.6% (6/41)	0% (0/41)

Data adapted from Shionogi & Co., Ltd., 1st Half of Fiscal 2019 Financial Results.

Patients with paired baseline and follow-up RT-PCR-positive samples evaluable for Sanger Sequencing (http://www.shionogi.co.jp/en/ir/pdf/e_p191031.pdf).

(PA) Polymerase acidic protein.

during the 2018–2019 influenza season showed similar replication fitness and pathogenicity to those of wild-type isolates in hamsters and transmitted efficiently between ferrets by respiratory droplets (Imai et al. 2020). These results, together with human-to-human transmission cases in Japan, indicate that currently circulating viruses have already acquired amino acid substitutions to compensate for the fitness costs of the PA I38T substitution. Because of the limited data and high frequency of variants with reduced susceptibility to baloxavir, the Japanese Association for Infectious Diseases and the Japan Pediatric Society have not actively recommended use of baloxavir for treatment of influenza in children younger than 12 yr and have not recommended use of baloxavir for monotherapy of influenza in the severely immunosuppressed.

The emergence of antiviral resistance is a great concern. Evidence suggests that the detection of the PA I38T substitution can be considered a laboratory correlate of clinically relevant baloxavir resistance (Gubareva and Fry 2019). Because global surveillance of baloxavir resistance is essential, the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) (https://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/en/) has initiated a global analysis of circulating influenza viruses in the 2017–2018 influenza season (Takashita et al. 2020). The monitoring of baloxavir susceptibility is important for public health planning purposes and for making clinical recommendations for antiviral use.

Table 4. Influenza viruses with I38 substitutions in PA detected in the 2018–2019 influenza season in Japan

Type/subtype	Total frequency	Age group (years)					
		0–5	6–11	12–19	20–64	≥65	Unknown
A(H1N1)pdm09	2.3% (9/395)	0% (0/61)	3.4% (7 ^a /203)	2.3% (1/44)	1.6% (1 ^a /63)	0% (0/21)	0% (0/3)
A(H3N2)	8.0% (34/424)	8.9%	12.5% (16 ^a /128)	9.6% (5 ^a /52)	1.1% (1/90)	5.6% (2 ^a /36)	0% (0/6)
			(10 ^a /112)				
B	0% (0/44)	0% (0/9)	0% (0/16)	0% (0/8)	0% (0/7)	0% (0/3)	0% (0/1)

(PA) Polymerase acidic protein.

^aThe I38 mutant viruses isolated from patients without prior baloxavir treatment were included.

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