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Treatment-Emergent Influenza Variant Viruses With Reduced Baloxavir Susceptibility: Impact on Clinical and Virologic Outcomes in Uncomplicated Influenza

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Background. Single-dose baloxavir rapidly reduces influenza virus titers and symptoms in patients with uncomplicated influenza, but viruses with reduced in vitro susceptibility due to amino acid substitutions at position 38 of polymerase acidic protein (PA/I38X) sometimes emerge.

Methods. We evaluated the kinetics, risk factors, and effects on clinical and virologic outcomes of emergence of PA/I38X-substituted viruses.

Results. Viruses containing PA/I38X substitutions were identified 3–9 days after baloxavir treatment in 9.7% (36/370) of patients, of whom 85.3% had transient virus titer rises. Median time to sustained cessation of infectious virus detection was 192, 48, and 96 hours in the baloxavir recipients with PA/I38X-substituted viruses, without PA/I38X-substituted viruses, and placebo recipients, respectively. The corresponding median times to alleviation of symptoms were 63.1, 51.0, and 80.2 hours, respectively. After day 5, symptom increases occurred in 11.5%, 8.0%, and 13.0%, respectively, and in 8.9% of oseltamivir recipients. Variant virus emergence was associated with lower baseline neutralizing antibody titers.

Conclusions. The emergence of viruses with PA/I38X substitutions following baloxavir treatment was associated with transient rises in infectious virus titers, prolongation of virus detectability, initial delay in symptom alleviation, and uncommonly with symptom rebound. The potential transmissibility of PA/I38X-substituted viruses requires careful study.

Clinical Trial Registration. NCT02954354.

Keywords. antiviral susceptibility; baloxavir marboxil; cap-dependent endonuclease; influenza; polymerase acidic protein.

RNA viruses, such as influenza, evolve rapidly due primarily to the error-prone nature of viral RNA-dependent RNA polymerase (RdRpol) [1–4]. The resulting genetic diversity in circulating influenza viruses can change many functional aspects including antigenicity, pathogenicity, transmissibility, or susceptibility to antivirals [1]. Seasonal influenza viruses resistant to the adamantanes circulate widely [5, 6], and therefore the adamantanes are no longer recommended for the influenza treatment [5, 7]. The most widely used anti-influenza drugs are neuraminidase inhibitors (NAIs), particularly oseltamivir [8]. Oseltamivir-resistant seasonal A(H1N1) viruses, containing an amino acid substitution at position 275 in the NA protein (NA/H275Y) [9], emerged in 2007 and circulated worldwide in 2008–2009, until being replaced by the 2009 A(H1N1) pandemic virus [9, 10]. Although the NA/H275Y substitution was previously shown to reduce virus transmissibility [11–13], enabling secondary NA substitutions in the 2007 A(H1N1) viruses were responsible for restoring fitness in the viruses, such that they were able to circulate in the absence of selective drug pressure [14, 15], and replaced oseltamivir-susceptible seasonal A(H1N1) viruses [16–18]. Since that time, the frequency of NAI-resistant A(H1N1)pdm09 viruses circulating in the community has remained below 4% [19], but there is concern about the potential for further oseltamivir-resistance emergence. Furthermore, outbreaks and pandemics by avian and other zoonotic influenza viruses are significant public health concerns. Therefore, additional anti-influenza drugs with novel mechanisms of action and potent antiviral effect against wide ranges of influenza virus are needed [20].

Cap-dependent endonuclease (CEN) is part of the polymerase acidic (PA) protein within the RdRpol complex of influenza A and B viruses. Baloxavir marboxil (formerly S-033188;

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hereafter baloxavir) is a prodrug of baloxavir acid (BXA), a CEN inhibitor approved in Japan and the United States for the treatment of influenza virus type A and B infections [21], following randomized trials in otherwise healthy adults and adolescents [22, 23]. In the phase 3 trial (CAPSTONE-1), a single oral dose of baloxavir rapidly reduced infectious virus titers and significantly reduced the time to alleviation of symptoms (TTAS) compared with placebo [22].

A preclinical study [24] determined that sequential passage of influenza A viruses in vitro selected variant viruses harboring amino acid substitutions from isoleucine (I) to threonine (T), and clinical studies [22] identified substitution to threonine (T), methionine (M), or phenylalanine (F) at position 38 of the PA (PA/I38X), within the active site of CEN [25–27]. In vitro characterization showed that laboratory strains with PA/I38X substitutions have reduced susceptibility to BXA [24], but also reduced endonuclease activity and impaired replicative fitness in cell culture [28, 29]. PA/I38X substitutions increased the BXA concentration achieving 50% inhibition of plaque formation (EC_{50}) of type A by 30- to 50-fold and type B viruses by 7-fold, with the I38T substitution having the largest effect [29]. Notably, PA/I38X-substituted viruses remain fully susceptible to oseltamivir [29].

During posttreatment monitoring of the phase 2 and 3 trials, PA/I38X-substituted viruses were identified in 2.2%–9.7% of baloxavir-treated patients [22]. Limited analyses of the phase 3 data found that baloxavir recipients with emergence of PA/I38X-substituted viruses had prolonged detectability of infectious virus and of TTAS compared to those who did not (median, 63.1 hours versus 49.6 hours). However, the impact of the emergence of PA/I38X-substituted viruses on the clinical and virologic effectiveness of baloxavir, as well as the susceptibility of variant clinical isolates, remain to be fully characterized. The aims of our post hoc analyses were to evaluate (1) the relationship between emergence of PA/I38X-substituted viruses and clinical and virologic outcomes in baloxavir-treated patients with uncomplicated influenza, (2) the kinetics of their emergence, (3) the changes in phenotypic susceptibility conferred by the PA/I38X substitutions in clinical isolates, and (4) the possible risk factors associated with the emergence of viruses with PAI38X substitutions.

MATERIALS AND METHODS

Study Design

CAPSTONE-1 was a phase 3, double-blind, placebo- and active comparator-controlled, randomized trial conducted in Japan and the US between in 2016/17 influenza season [22] (ClinicalTrials.gov NCT02954354). The methods, including sample collection, virologic analyses, and primary results of the trial have been reported [22]. Next-generation sequencing (NGS) of swabs and phenotypic assays of virus susceptibility were conducted in selected patients (Supplementary Text).

Risk Factors and Relationship to Drug Exposure

The possible enrollment risk factors assessed for emergence of PA/I38X-substituted viruses included sex, body weight, composite symptom score, body temperature, neutralizing antibody titer, infectious virus titer, time to treatment from symptom onset, food before or after administration, and medical history. Age and influenza vaccination were excluded because they had statistically significant associations with baseline neutralizing antibody titer. Region was excluded because it had a statistically significant association with time to treatment from symptom onset (Supplementary Table 1).

Plasma samples were taken on days 2 and 5, and when possible on days 1 (0.5 to 4 hours postdose), 3, and 15. The BXA concentrations at 24 hours (C_{24}; from 20 to 28 hours postdose) and 72 hours postdose (C_{72}) and area under the curve (AUC) were used for the assessments. C_{72} and AUC were estimated by a Bayesian approach based on the population pharmacokinetic model [30, 31].

Outcomes and Statistical Analyses

The primary efficacy outcome was the TTAS [22]. Also, we examined the time to alleviation of fever, symptoms score over time, and the proportions of participants who reached the alleviation endpoint but then had recurrence of at least 1 symptom self-rated as moderate (score = 2) or greater in intensity. The virology outcomes were the time to sustained cessation of infectious virus detection (time between the start of treatment and when virus titer remained below the detection limit on all subsequent sampling time points).

Efficacy outcomes were analyzed in the intention-to-treat infected population, defined as randomized patients with influenza confirmed by reverse transcription polymerase chain reaction (RT-PCR) on day 1, who received study drug. In our post hoc analyses, baloxavir recipients were divided into 2 subgroups: those with and those without detectable PA/I38X-substituted viruses postdose. For the TTAS and time to resolution of fever, median durations with 95% confidence intervals (CI) were calculated, and Kaplan–Meier curves were plotted. Statistical comparison between baloxavir- and placebo-treated groups was performed using generalized Wilcoxon test with stratification factors (baseline composite symptom score and region).

For patients infected with A(H3N2) virus, risk factors associated with emergence of PA/I38X-substituted viruses were identified using a logistic regression model.

All post hoc analyses were performed using SAS Version 9.2.

RESULTS

Treatment-Emergent PA/I38X-Substituted Viruses

No PA/I38X substitutions were observed in viruses at baseline or from placebo-treated patients (n = 95). Of 456 baloxavir-treated, influenza-infected patients, 370 (81.1%) had paired

baseline and follow-up RT-PCR–positive samples evaluable for Sanger sequencing [22]. Among these patients, PA/I38X-substituted viruses were observed in 36, representing 7.9% of all baloxavir-treated, influenza-infected patients and 9.7% of those with paired sequence data. Those with substituted viruses included 35 with A(H3N2) infection (PA/I38T [n = 30], PA/I38T/I mixture [n = 2], PA/I38T/I/M mixture [n = 2], and PA/I38I/M mixture [n = 1]), and 1 coinfection with A(H3N2) and type B (PA/I38T/I mixture in both viruses) (Supplementary Table 2).

BXA susceptibility testing of 5 pairs of isolates determined that the range of BXA EC_{50} values was 0.31–0.69 ng/mL in the pretreatment (PA/I38) and 36–63 ng/mL in the posttreatment samples (PA/I38T), while the favipiravir susceptibility of PA/I38T-substituted viruses remained unchanged (Table 1 and Supplementary Figure 1).

For patients infected with A(H3N2), the last evaluable positive sample containing PA/I38X-substituted viruses was on day 4 (1 case), 5 (27 cases), 6 (2 cases), and 9 (5 cases). For 1 patient with A(H3N2) and type B coinfection, the last evaluable positive samples containing PA/I38X-substituted viruses were on days 3 (A(H3N2)) and 6 (type B). The mean (SD) decline of infectious viral titer from baseline to day 2 was −5.01 (1.65) $\log_{10}$ $TCID_{50}$/mL in baloxavir recipients with PA/I38X-substituted viruses and −4.60 (1.97) $\log_{10}TCID_{50}$/mL in those without, compared to −1.19 (2.43) $\log_{10}TCID_{50}$/mL in placebo recipients ($TCID_{50}$: 50% tissue culture infectious dose).

To more precisely assess the timing when PA/I38X-substituted viruses first emerged, an analysis by NGS was conducted on samples collected on multiple consecutive days from 7 patients with PA/I38X-substituted viruses (Supplementary Figure 2). In the pretreatment baseline samples, no PA/I38X substitutions were detected (Figure 1A). PA/I38T substitutions were found to have emerged as early as day 3 in 2 patients; in 2 other patients the variant viruses emerged as a mixed viral population by day 4; and in 3 patients variant viruses emerged by day 5 (Figure 1A). The PA/I38T-substituted viruses became the dominant population in all 7 patients and in each the timing of PA/I38T-substituted virus emergence corresponded with an increase in viral titers, which in most cases had been below infectivity assay detection limits for at least 1 day prior to the emergence of variant viruses (Figure 1B). No obvious differences in plasma BXA concentrations were noted in these 7 patients, although both with day 3 emergence had relatively lower concentrations over time (Figure 1C).

Relationship of Variant Viruses Emergence to Cessation of Viral Shedding

In baloxavir recipients with paired sequence data, the median time to sustained cessation of infectious virus detection was 192 hours (95% CI, 168.0–192.0) for those with PA/I38X-substituted viruses (n = 34) and 48 hours (CI not estimated) for those without substituted viruses (n = 325), compared with 96 hours (95% CI, 96.0–120.0) for placebo recipients (n = 209). In 85.3% of the baloxavir-treated subgroup with PA/I38X-substituted viruses, infectious virus titer increased transiently, particularly from days 3 to 6 (Figure 2). The increases were as high as 5.5 $\log_{10}TCID_{50}$/mL in some cases, but remained low (ie, <2.0 $\log_{10}TCID_{50}$/mL) in most. The mean (SD) viral titer on day 5 in patients with PA/I38X-substituted viruses was 2.33 $\log_{10}TCID_{50}$/mL (1.20) compared with 1.13 $\log_{10}TCID_{50}$/mL (1.05) for placebo patients. On day 5, infectious virus was still detectable in 27/30 (90.0%) of baloxavir-treated patients with PA/I38X-substituted viruses, compared to 26/313 (8.3%) of baloxavir-treated patients without PA/I38X-substituted viruses, 57/192 (29.7%) of patients receiving placebo, and 70/336 (20.8%) of patients receiving oseltamivir (20 to 64 years of age).

Table 1. Susceptibility of Influenza PA/I38T-Substituted Viruses Propagated From Clinical Samples to Baloxavir Acid and Favipiravir

Patient ID	Pre/Post Baloxavir Sample (PA/38 Amino Acid)	Baloxavir Acid		Favipiravir	
		Mean EC_{50}, ng/mL (SD)	Fold Change	Mean EC_{50} ng/mL (SD)	Fold Change
186102	Pre (WT)	0.46 (0.28)	NA	3000 (1100)	NA
	Post (PA/I38T)	63[a]	136	3600[a]	1.2
253104	Pre (WT)	0.69 (0.32)	NA	2900 (510)	NA
	Post (PA/I38T)	53[a]	77	3800[b]	1.3
297105	Pre (WT)	0.56 (0.35)	NA	2200 (870)	NA
	Post (PA/I38T)	36 (15)	65	2900 (1300)	1.3
339111	Pre (WT)	0.31 (0.12)	NA	2200 (720)	NA
	Post (PA/I38T)	49 (22)	155	2500 (930)	1.1
344103	Pre (WT)	0.39 (0.06)	NA	1700 (420)	NA
	Post (PA/I38T)	36 (11)	93	2400 (1100)	1.4

Data represent mean and SD of 3 independent experiments, each performed in duplicate unless otherwise indicated. Fold change was calculated by dividing the mean EC_{50} of the post sample by the mean EC_{50} of the cognate pre sample.

Abbreviations: EC_{50}, concentration achieving 50% inhibition of plaque formation; I, isoleucine; ID, identity; NA, not applicable; PA/38, position 38 of virus polymerase acidic protein; T, threonine; WT, wild type.

[a]Mean of 1 experiment performed in duplicate.

[b]Value of a single replicate from a single experiment.

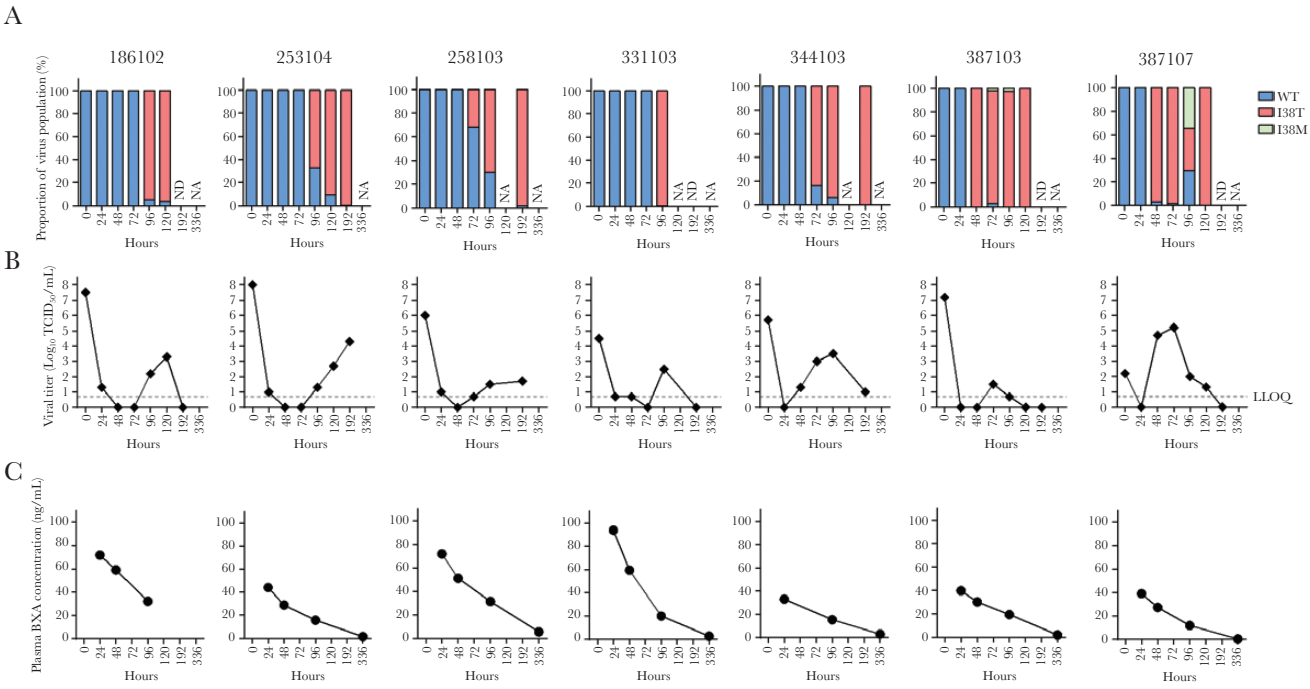


Figure 1. Proportion of variant viruses with PA/I38X substitutions, viral titer and plasma BXA concentration in the swab samples from selected patients. Time courses of % proportion of PA/I38X-substituted viruses in the swabs (*A*), viral titer ($log_{10}TCID_{50}$/mL) in the swabs (*B*), and BXA concentration in plasma (ng/mL) after day 2 (*C*) are shown. The patient identity number is indicated above. A threshold frequency of >1% was adopted for calling variant viruses in next generation sequencing analysis. The lower limit of quantification of viral titer was set at 0.7 $log_{10}TCID_{50}$. Abbreviations: BXA, baloxavir acid; PA/I38T, isoleucine substituted by threonine at position 38 of virus polymerase acidic protein; PA/I38M, isoleucine substituted by methionine at position 38 of virus polymerase acidic protein; WT, wild-type virus; ND, below quantitation limit of 2.18 log_{10}RNA copies/mL; NA, not applicable; $TCID_{50}$, 50% tissue culture infectious dose.

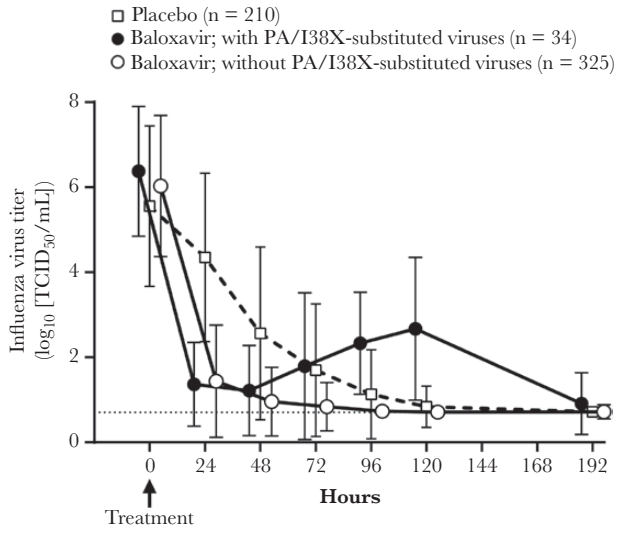
On day 9, infectious virus remained detectable in 5/34 (14.7%) of baloxavir, with PA/I38X-substituted viruses, 6/309 (1.9%) of baloxavir, without PA/I38X-substituted viruses, 9/197 (4.6%) of placebo, and 11/340 (20.8%) of oseltamivir.

Relationship of Variant Viruses Emergence to Clinical Outcomes

Overall, the TTAS in baloxavir recipients who had paired sequence data was significantly shorter than those receiving placebo treatment (difference, −26.8 hours, $P < .001$; Supplementary Table 3). The median TTAS in the baloxavir-treated subgroup with PA/I38X-substituted viruses (63.1 hours; 95% CI, 52.2–87.7) was 12.0 hours longer than in the baloxavir-treated subgroup without PA/I38X-substituted viruses (51.0 hours; 95% CI, 46.0–56.0), but 17.2 hours shorter than in the placebo group (80.2 hours; 95% CI, 72.6–87.1) (Figure 3). The median TTAS in the oseltamivir group (20 to 64 years of age) was 53.8 hours (95% CI, 50.2–56.4) [22]. Differences in the proportions with symptom alleviation between the baloxavir-treated subgroups began after 24 hours, but after approximately 60 hours the proportions of unalleviated patients were similar between baloxavir-treated patients with or without PA/I38X-substituted viruses (Figure 3). No significant differences in symptom scores over time were found between the baloxavir subgroups, and no late increases in score were noted in those with PA/I38X-substituted viruses (Figure 4). The proportion of patients who had symptom alleviation before day 5 and subsequently experienced increased influenza symptoms after day 5 was similar across the baloxavir subgroup with PA/I38X-substituted viruses (3/26; 11.5%), baloxavir subgroup without PA/I38X-substituted viruses (20/249; 8.0%), placebo group (19/146; 13.0%), and oseltamivir group (20 to 64 years of age) (26/291; 8.9%) (Supplementary Table 4 and Table 5). The corresponding median durations of fever were 31.0 hours (95% CI, 23.8–33.0), 24.4 hours (95% CI, 22.1–26.5), 42.0 hours (95% CI, 37.4–44.6), and 24.0 hours (95% CI, 22.1–25.9), respectively (Supplementary Figure 3 and Supplementary Tables 6–8).

Risk Factors for Emergence

The distribution of baseline demographic and other characteristics in baloxavir-treated patients with or without PA/I38X-substituted viruses are shown in Table 2. A greater proportion of baloxavir-treated patients from Japan (33/266; 12.4%) had PA/I38X-substituted viruses compared with patients from the United States (2/61; 3.3%), and a greater proportion of baloxavir-treated patients with low neutralizing antibody titer had emergence of PA/I38X-substituted viruses compared with patients with high antibody titer. Multivariate analysis identified a statistically significant association between baseline neutralizing virus antibody titer and emergence of



No. of Patients

Baloxavir; with PA/I38X-substituted viruses	34	34	34	7	30	6	-	-	34
Baloxavir; without PA/I38X-substituted viruses	325	316	308	89	310	72	-	-	308
Placebo	210	201	191	57	192	48	-	-	197

Figure 2. Time course of influenza virus titer (mean ± SD) in baloxavir-treated patients with and without treatment-emergent PA/I38X-substituted viruses and in placebo-treated patients. The lower limit of quantification of viral titer was set at 0.7 $log_{10}TCID_{50}$ (dotted line). The subset of patients who were positive for infectious virus titer at baseline was included in this analysis; 2 baloxavir-treated patients with PA/I38X-substituted viruses had negative baseline virus titer (individual data shown in Supplementary Figure 5). Abbreviations: PA/I38X, amino acid substitutions of isoleucine at position 38 of virus polymerase acidic protein; $TCID_{50}$, 50% tissue culture infectious dose.

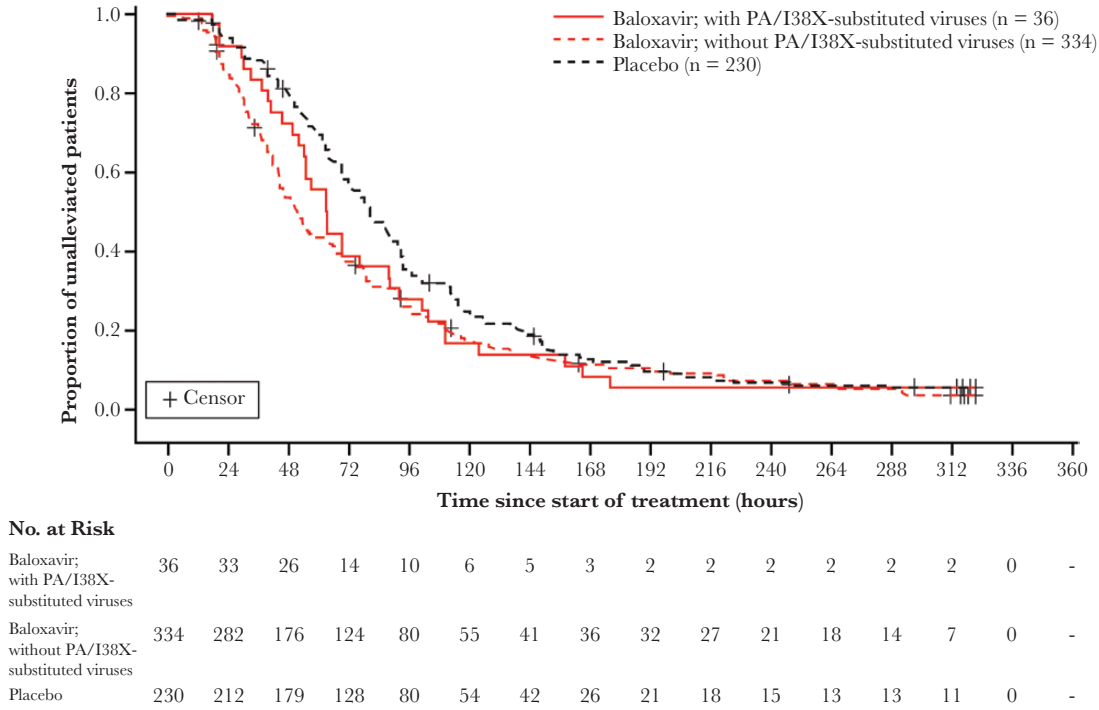
PA/I38X-substituted viruses. However, no significant associations were found with other factors (Table 3). Although the C_{24} and extrapolated AUC and C_{72} in baloxavir recipients were numerically higher in the Japanese subgroup compared to the non-Japanese subgroup, the baloxavir subgroups with and without PA/I38X-substituted viruses showed comparable BXA exposures within the Japanese or American subgroups (Supplementary Figure 4).

DISCUSSION

In this phase 3 randomized, controlled trial of baloxavir in otherwise healthy adults and adolescents, we detected treatment-emergent PA/I38X-substituted viruses in 7.9% of all baloxavir-treated, influenza-infected patients, including 9.7% of those with paired sequence data and 10.9% of those with A(H3N2) infection. Emergence of variant viruses occurred as early as day 3 and was associated with transient rises in infectious virus titers and prolongation of virus detectability. PA/I38X-substituted viruses emergence was also associated with an impact on illness resolution in uncomplicated influenza. We found an initial slowing of illness resolution in those with subsequent detection of PA/I38X-substituted viruses. The initial delay in alleviation of symptoms in the baloxavir subgroup with PA/I38X-substituted viruses was associated with a 12-hour longer TTAS compared to the subgroup without PA/I38X-substituted viruses. However, symptom alleviation from 60 hours (2.5 days) onwards after baloxavir treatment was comparable, as were influenza symptom scores over time for the 2 subgroups (Figure 4). Also, the proportion of patients with recurrence of influenza symptoms or fever after reaching the TTAS showed no significant differences across the treatment groups. Of note, the initial delay in illness alleviation occurred before the PA/I38X-substituted viruses and associated increases in viral titers rises were detected. In comparison, prior observations in oseltamivir-treated adult and pediatric outpatients with uncomplicated influenza found that those with emergence of oseltamivir-resistant variant viruses, mostly children, had prolongation of viral RNA detectability although illness duration was not longer compared to those without emergence of such variants [32, 33].

The reasons why alleviation of influenza symptoms was initially delayed as early as 24 hours posttreatment in the patient population shedding PA/I38X-substituted viruses are not clear. The initial decreases in viral titer were similar in the baloxavir subgroups, and the PA/I38X-substituted viruses were not detectable in patient samples prior to day 3. However, this raises the possibility that host factors may have played a role in slower symptom resolution and emergence of PA/I38X-substituted viruses. In this regard we found that lower baseline neutralizing antibody titer was significantly associated with higher frequency of PA/I38X-substituted virus emergence. In such patients with low baseline antibody titers, variant viruses with reduced susceptibility may have emerged due to limited host immune responses at the time of waning plasma BXA concentrations (see below). However, we did not examine early innate immune or antibody responses during the first 3–5 days in our study. In any case, the possibility that emergence of PA/I38X-substituted viruses might be associated with prolongation of virus replication and influenza illness in important risk populations (eg, infants and children, hospitalized patients, and immunocompromised hosts) will require careful study.

PA/I38X-substituted viruses were not present in any patient at baseline, consistent with evidence that circulating viruses with PA/I38X substitutions have been very rare to date [29, 34]. In the phase 2 trial, treatment-emergent PA/I38X-substituted viruses were detected in 2.2% of patients with paired sequence data (1.3% of all baloxavir-treated, influenza-infected patients), all of whom were infected with A(H1N1)pdm09 viruses [22]. Treatment-emergent influenza viruses with reduced susceptibility have been observed with both adamantanes and NAIs. The frequency of resistant viruses post-rimantadine treatment was 33% in adults, during a period when adamantane-susceptible viruses were circulating [35]. Furthermore, the M2/S31N substitution conferring adamantane resistance does not confer a fitness loss and now persists in more than 95%



No. at Risk

Baloxavir; with PA/I38X-substituted viruses	36	33	26	14	10	6	5	3	2	2	2	2	2	2	0	-
Baloxavir; without PA/I38X-substituted viruses	334	282	176	124	80	55	41	36	32	27	21	18	14	7	0	-
Placebo	230	212	179	128	80	54	42	26	21	18	15	13	13	11	0	-

Figure 3. Kaplan–Meier curve of time to alleviation of symptoms in baloxavir-treated patients with and without treatment-emergent PA/I38X-substituted viruses and in placebo-treated patients. Patients who did not experience alleviation of symptoms were censored at the last observation time point. Abbreviation: PA/I38X, amino acid substitutions of isoleucine at position 38 of virus polymerase acidic protein.

of currently circulating influenza A viruses among humans [36]. In prospective studies with oseltamivir, the frequency of treatment-emergent oseltamivir-resistant virus was approximately 1% in outpatient adults [32]. The current study did not test for oseltamivir resistance. A comparison of the frequencies of treatment-emergent viruses with reduced susceptibility across studies is difficult because of varying assay methods and monitoring approaches used to identify variant viruses [37, 38], as well as differences in circulating strains. However, the available data indicate the frequency of PA/I38X-substituted viruses is higher in A(H3N2)-infected patients following single-dose baloxavir treatment than the frequency of oseltamivir resistance seen with oseltamivir treatment [32].

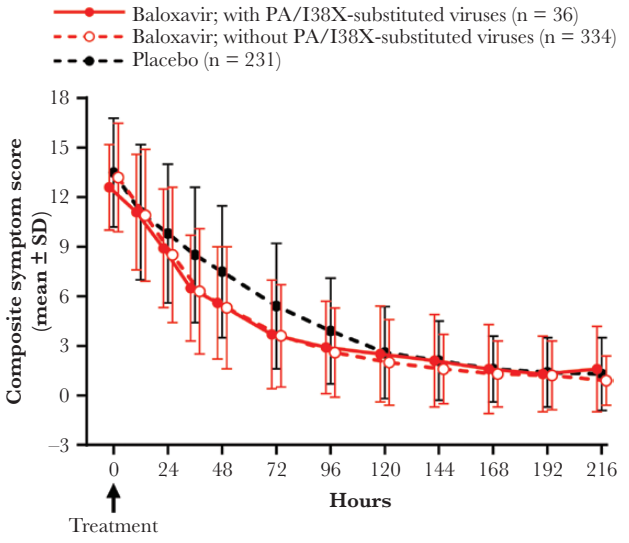


Figure 4. Composite score (mean ± SD) in baloxavir-treated patients with and without treatment-emergent PA/I38X-substituted viruses and in placebo-treated patients. Abbreviation: PA/I38X, amino acid substitutions of isoleucine at position 38 of virus polymerase acidic protein.

Due to a lack of proofreading activity, influenza viruses have a high gene mutation rate resulting in approximately 1 error per replicated genome [39]. Nonsynonymous mutations can lead to variant viruses evading either host antibody responses (if substitutions occur in HA antibody binding sites) or antiviral drug pressure (if substitutions arise in drug binding sites). The error-prone properties of the virus mean that mutations, like those that lead to PA/I38X substitutions, will inevitably occur during viral replication, such that under selective baloxavir treatment the PA/I38X-substituted viruses may become dominant in a viral population due to their replication advantage over baloxavir-sensitive wild-type viruses in some patients (Figure 1). In our study, most patients with PA/I38X-substituted viruses were Japanese, who had somewhat higher plasma BXA levels compared to American patients due to ethnic differences in BXA exposure [40]. However, we did not see any clear differences in plasma BXA concentrations in

Table 2. Baseline Characteristics of Baloxavir-Treated Patients With A(H3N2) Virus Infection and Patients With and Without Treatment-Emergent PA/I38X-Substituted Viruses

Characteristic	Baloxavir Marboxil		
	Patients With PA/I38X-Substituted Viruses (n = 35)	Patients Without PA/I38X-Substituted Viruses (n = 292)	Total (n = 327)[a]
Age, y, median (range)	32.0 (13–64)	31.0 (12–64)	31.0 (12–64)
Weight			
Mean, kg (SD)	58.26 (12.26)	64.90 (14.75)	64.19 (14.63)
<80 kg, n (%)	34 (97.1)	244 (83.6)	278 (85.0)
BMI, kg/m^2, mean (SD)	21.63 (3.73)	23.39 (4.39)	23.20 (4.35)
Sex, male, n (%)	14 (40.0)	156 (53.4)	170 (52.0)
Region, n (%)			
Japan	33 (94.3)	233 (79.8)	266 (81.3)
United States	2 (5.7)	59 (20.2)	61 (18.7)
Composite symptom score at baseline, mean (SD)	12.60 (2.60)	13.16 (3.28)	13.10 (3.21)
Body temperature at baseline, °C, mean (SD)	38.42 (0.55)	38.50 (0.55)[b]	38.49 (0.55)[c]
Infectious virus titer at baseline, $\log_{10}$TCID$_{50}$/mL, mean (SD)	6.23 (1.81)[d]	6.04 (1.63)[e]	6.06 (1.65)[f]
Time to treatment from symptom onset, n (%)			
0 to ≤12 hours	6 (17.1)	38 (13.0)	44 (13.5)
>12 to ≤24 hours	16 (45.7)	120 (41.1)	136 (41.6)
>24 to ≤36 hours	9 (25.7)	89 (30.5)	98 (30.0)
>36 to ≤48 hours	4 (11.4)	45 (15.4)	49 (15.0)
Influenza vaccination, n (%)	9 (25.7)	79 (27.1)	88 (26.9)
Neutralizing antibody titer, n (%)			
<20	13 (37.1)	51 (17.6)	64 (19.8)
≥20	22 (62.9)	238 (82.4)	260 (80.2)
Meal before administration, n (%)			
Yes	22 (62.9)	176 (60.3)	198 (60.6)
No	13 (37.1)	116 (39.7)	129 (39.4)
Time from meal before administration, n (%)			
<2 hours	1 (4.5)	6 (3.4)	7 (3.5)
≥2 to ≤4 hours	9 (40.9)	51 (29.0)	60 (30.3)
>4 hours	12 (54.5)	119 (67.6)	131 (66.2)
Meal after administration, n (%)			
Yes	32 (91.4)	237 (81.2)	269 (82.3)
No	3 (8.6)	54 (18.5)	57 (17.4)
Missing data	0	1 (0.3)	1 (0.3)
Time until meal after administration, n (%)			
<2 hours	16 (50.0)	112 (47.3)	128 (47.6)
≥2 to ≤4 hours	8 (25.0)	61 (25.7)	69 (25.7)
>4 hours	8 (25.0)	64 (27.0)	72 (26.8)

Abbreviations: BMI, body mass index; PA/I38X, amino acid substitutions of isoleucine at position 38 of virus polymerase acidic protein; TCID$_{50}$, 50% tissue culture infectious dose.

[a]Intention-to-treat infected population population with A(H3N2) virus infection and paired baseline and follow-up reverse transcription polymerase chain reaction-positive samples evaluable for Sanger sequencing.

[b]n = 290.

[c]n = 325.

[d]n = 34.

[e]n = 284.

[f]n = 318.

Japanese patients with PA/I38X-substituted viruses compared to those without (Supplementary Figure 4). While potentially contributory, BXA exposure differences are insufficient to explain the selection of PA/I38X-substituted viruses in our study, and further pharmacokinetic-pharmacodynamic studies in larger populations are required.

Notably, PA/I38X-substituted viruses tended to emerge at days 3–5 along with decreasing plasma BXA concentrations. The C_{72} estimated plasma BXA concentration was approximately 30 ng/mL (62 nmol/L) in both subgroups with and without PA/I38X-substituted viruses. This concentration is about 54- to 75-fold higher than the EC_{50} against wild-type

Table 3. Identification of Prognostic Factors for Emergence of PA/I38X-Substituted Viruses by Logistic Regression Analysis

Parameter	Odds Ratio	95% CI	*P* Value
Sex, male vs female	0.715	0.308–1.665	.4370
Baseline body weight, by 1 kg	0.967	0.933–1.001	.0603
Baseline composite symptom score, by 1 score	0.943	0.838–1.061	.3330
Baseline body temperature, by 1 degree	0.572	0.269–1.217	.1469
Baseline neutralizing virus antibody titer, ≥20 to <20	0.335	0.150–0.751	.0079
Baseline influenza virus titer, by 1 $log_{10}TCID_{50}$/mL	1.049	0.830–1.327	.6883
Time to treatment from symptom onset, by 12 hours[a]	0.740	0.481–1.139	.1715
Meal before administration on day of dosing, yes vs no	1.089	0.499–2.377	.8307
Meal after administration on day of dosing, yes vs no	1.598	0.435–5.875	.4802
Medical history, yes vs no[b]	0.823	0.378–1.792	.6236

A total of 312 baloxavir-treated patients infected with influenza A(H3N2) virus who had paired sequencing data available and had baseline characteristic data for possible risk factor were included in this analysis.

Abbreviations: CI, confidence interval; PA/I38X, amino acid substitutions of isoleucine at position 38 of virus polymerase acidic protein; $TCID_{50}$, 50% tissue culture infectious dose.

[a]Treated as ordered category of 4 levels (≥0 to ≤12 hours, >12 to ≤24 hours, >24 to ≤36 hours, >36 to ≤48 hours).

[b]Previous or concurrent significant condition such as hospitalization, all concurrent medical conditions, and surgical history within 12 months.

recombinant influenza A(H3N2) virus in vitro (0.40 to 0.56 ng/mL), but quite close to the EC_{50}s for laboratory strains [29] and for the clinical isolates with PA/I38T substitutions in this study (36 to 63 ng/mL). Thus, at days 3–5, plasma BXA concentrations would be near or below the EC_{50} for PA/I38T-substituted viruses, but still well above the EC_{50} for wild-type virus. Indeed, in 7 patients with NGS and pharmacokinetic data, PA/I38T-substituted viruses were first observed when the plasma BXA concentration was 15.4 to 32.2 ng/mL (Figure 1), indicating that plasma BXA concentrations had decreased to levels for which decreased inhibition of PA/I38T virus replication is seen in vitro. Our observations indicate that alternative dosing regimens or treatment approaches (eg, repeat baloxavir dosing at 48–72 hours or combination with NAI) warrant study to reduce the risk of variant viruses emergence, especially in patients with greater likelihood of prolonged viral replication (eg, young children, seriously ill, hospitalized, or immunocompromised).

Among the strengths of our study are that our results are complemented by data on BXA pharmacokinetics [40] and that changes in phenotypic susceptibility conferred by the PA/I38T substitutions in clinical isolates were confirmed to be comparable to those generated in laboratory strains. We directly genotyped clinical specimens both before and after baloxavir treatment, without an intervening propagation step [29], and used nested PCR sequencing to effectively lower the level of detection, allowing identification of variant viruses later in the course of infection when virus titers are relatively low. However, we first screened viruses for PA substitutions genotypically by Sanger sequencing, and therefore we may have missed minor variant viruses and may not have detected them at the other time points or changes in other viral genes. Furthermore, analyses for assessing clinical impact of PA/I38X-substituted viruses were not prespecified in the protocol, and the study was not adequately powered to assess risk factors in the baloxavir subgroups with and without PA/I38X-substituted viruses. The trial included patients from Japan and the United States during the 2016–2017 season who were primarily infected with A(H3N2) virus, and the frequency of PA/I38X-substituted virus emergence may differ for other patient populations with more prolonged viral replication, seasons, or types/subtypes of influenza virus. In this regard we found less variant viruses emergence in uncomplicated influenza A(H1N1) and type B virus infections [22].

The transmissibility of PA/I38X-substituted viruses is currently unknown and needs assessment to understand the risk that such viruses may spread amongst the community in the absence of drug pressure [41]. Although their replicative fitness in vitro is reduced [28, 29], it is possible that secondary permissive mutations could restore infectivity and transmissibility, as has been observed for some NAI- and adamantane-resistant viruses [5, 9, 16]. The rebound in infectious viral titer and prolonged virus detection in baloxavir recipients with PA/I38X-substituted viruses beyond what was seen in the placebo group does suggest that these variant viruses retain at least some level of fitness. Studies of the replicative capacity and transmissibility of PA/I38X-substituted viruses are currently underway in animal models, and a household-based clinical trial (JapicCTI-184180) is underway to explore the potential for human-to-human transmission of viruses from baloxavir-treated index cases. The potential transmissibility of PA/I38X-substituted viruses should be monitored carefully through surveillance studies.

In conclusion, the emergence of viruses with PA/I38X substitutions following baloxavir treatment was associated with transient rises in infectious virus titers, prolongation of virus detectability, initial delay in symptom alleviation, and uncommonly with symptom rebound.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. T. U., K. K., S. O., K. B., T. S., and S. P. are employed by Shionogi. K. T. is employed by and holds stock in Shionogi. F. G. H. reports consulting fees, paid to the Robert Ford Haitian Orphanage and School Foundation, from Cidara, Shionogi, Seqirus, and PrEP Biopharm; fees for serving on data and safety monitoring boards, paid to the University of Virginia, from GlaxoSmithKline, Celltrion, and Vaccitech; travel support from Shionogi; and serving as unpaid consultant to Cocrystal Pharma, Farmak, Fujifilm/Toyama Chemical/MediVector, GlaxoSmithKline, Janssen, MedImmune, Regeneron, resTORbio, Roche/Genentech, Vir Biotechnology, and Visterra. A. C. H. reports receiving research funding from Shionogi. M. D. d. J. reports serving on an advisory board and travel support and fees for serving on an independent data and safety monitoring board, paid to his institution, from Janssen; serving on an advisory board and receiving travel support, paid to his institution, from MedImmune and Shionogi; and serving on an independent data and safety monitoring board and receiving travel support, paid to his institution, from GlaxoSmithKline and Vertex Pharmaceuticals.

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