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Pharmacokinetic and pharmacodynamic analysis of baloxavir marboxil, a novel cap-dependent endonuclease inhibitor, in a murine model of influenza virus infection

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Background: Baloxavir acid, the active form of the orally available prodrug baloxavir marboxil, is a novel cap-dependent endonuclease inhibitor of influenza virus. Baloxavir marboxil has been shown to rapidly reduce virus titres compared with oseltamivir in clinical studies.

Objectives: We investigated the relationship between pharmacokinetic (PK) parameters and antiviral activity of baloxavir acid based on virus titre reduction in lungs of infected mice.

Methods: BALB/c mice infected with a sub-lethal dose of influenza A(H1N1), A(H1N1)pdm09, A(H3N2) or type B virus were treated on day 5 with oral baloxavir marboxil (0.5–50 mg/kg q12h), subcutaneous baloxavir acid (0.25–8 mg/kg/day), oseltamivir phosphate (5 or 50 eq mg/kg q12h) or other antivirals for 1 day. Lung virus titres were assessed 24 h after initial antiviral dosing. PK testing was performed at up to 24 h post-dosing of baloxavir marboxil or baloxavir acid in A/WSN/33-infected mice and the PK/pharmacodynamic (PD) relationship was evaluated for baloxavir acid.

Results: Oral baloxavir marboxil administration showed dose-dependent virus titre reductions in lungs of mice infected with the different types/subtypes of influenza viruses 24 h post-dosing. Baloxavir marboxil at 15 mg/kg q12h resulted in ≥100-fold and ≥10-fold reductions in influenza A and B virus titres, respectively, compared with oseltamivir phosphate. PK/PD analysis showed that the plasma concentration at the end of the dosing interval (C_τ) or the plasma concentration at 24 h after initial dosing (C_{24}) was the PK parameter predicting the virus titres at 24 h post-dosing of baloxavir acid.

Conclusions: PK/PD analysis of baloxavir acid based on virus titre reduction in this mouse model could be helpful in predicting and maximizing virological outcomes in clinical settings.

Introduction

The influenza virus replicates almost exclusively in epithelial cells of the human upper and lower respiratory tract. Influenza virus replication peaks at approximately 48 h after inoculation and thereafter declines, with symptom resolution within 10 days. There is no doubt that abundant virus propagation in the respiratory tract causes onset of influenza illness.[1–3] Neuraminidase inhibitors (NAIs), such as oseltamivir phosphate, zanamivir hydrate and peramivir trihydrate, have been licensed as influenza antivirals in several countries. Many clinical trials in otherwise healthy influenza patients, where patients received antivirals at the peak or decline phase of virus shedding, showed that NAIs could reduce virus titres in nasopharyngeal wash by approximately 1 log, shortening the time to alleviation of influenza symptoms.[4–7] These data

suggest that appropriate assessment of viral load reduction by influenza antivirals is important for estimating therapeutic outcomes in clinical settings.

To predict and maximize antiviral efficacy for patients, it is necessary to determine the optimal clinical dosing regimens based on both preclinical and clinical data.[8–10] To accomplish this, pharmacokinetic (PK) and pharmacodynamic (PD) studies have been conducted.[11–13] For NAIs, both an *in vitro* hollow-fibre infection system and *in vivo* infection models were used to establish PK/PD correlation with suppression of viral replication and with mouse survival, respectively.[14–17] Another study investigated the *in vivo* viral load change for PB2 inhibitors in a lethal influenza A virus infection murine model.[18]

The cap-dependent endonuclease (CEN) is an essential enzyme for virus transcription and resides in the N-terminal domain of the PA subunit of RNA polymerase. Baloxavir marboxil is a selective CEN inhibitor approved for the treatment of influenza A and B virus infections. The baloxavir marboxil prodrug is promptly and fully metabolized in humans into its active form, baloxavir acid,[19] which inhibits replication of influenza A and B viruses *in vitro* and *in vivo*.[20–24] In clinical trials, a single dose of baloxavir marboxil led to a profound decline in virus titres on nasopharyngeal swabs and faster alleviation of influenza symptoms in patients with uncomplicated infection.[25]

The aim of the present study was to characterize the relationship between PK parameters and the antiviral activity of baloxavir acid based on virus titre reduction in the lungs of mice infected with influenza A virus, in order to predict and maximize the antiviral efficacy of baloxavir marboxil.

Methods

Ethics

All mouse studies were conducted under applicable laws and guidelines and with the approval of the Shionogi Animal Care and Use Committee (approval numbers: S10017D, S14072D, S14080D, S14083D, S16013D and S16129D).

Compounds

Baloxavir marboxil and baloxavir acid were from Shionogi & Co., Ltd, oseltamivir phosphate and zanamivir hydrate were from Sequoia Research Products Ltd (Pangbourne, UK) and laninamivir octanoate and favipiravir were from Toronto Research Chemicals Inc. (Toronto, Canada) and PharmaBlock Sciences, Inc. (Nanjing, China), respectively. Baloxavir marboxil and favipiravir were suspended using an agate mortar and pestle in 0.5% (w/v) methyl cellulose and 0.4% (w/v) carboxymethyl cellulose aqueous solution, respectively. Oseltamivir phosphate was dissolved in 0.5% (w/v) methyl cellulose, and zanamivir hydrate and laninamivir octanoate were dissolved in saline. Baloxavir acid was dissolved in 10% (w/v) Tween 80 and 0.5% (w/v) vinylpyrrolidone-vinyl acetate copolymer in sodium carbonate-sodium hydrogen carbonate buffer under heating and the pH was adjusted to ∼9. For dosing, each solution or suspension was diluted with the same respective vehicle.

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were obtained from the European Collection of Cell Cultures (Wiltshire, UK), A/WSN/33 (H1N1) and A/Osaka/129/2009 (H1N1pdm09) strains were obtained from the Osaka Prefectural Institute of Public Health and A/Hong Kong/8/68 (H3N2) and B/Hong Kong/5/72 strains were obtained from ATCC (Manassas, VA, USA). The A/WSN/33-NA/H274Y strain was prepared at Shionogi & Co. All viruses were propagated in MDCK cells and stored at −80°C until use, and the infectious titres determined by standard $TCID_{50}$ assay. For measurement of lung virus titres, serial dilutions of lung homogenates were inoculated onto confluent MDCK cells as described previously.[26] The presence of cytopathic effects was determined microscopically and virus titres were calculated as $\log_{10}$ $TCID_{50}$/mL. When no cytopathic effect was observed in the lowest dilution (10-fold), the titre of undetected virus was defined as 1.5 $\log_{10}$ $TCID_{50}$/mL.

Animals

Specific pathogen-free 6-week-old female BALB/c mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used.

Murine model construction

Virus titres in the lungs of mice inoculated with A/WSN/33 at 0.25×, 0.5× or 1× LD_{50} (five per group) were assessed over time to ascertain the time course of infection and to determine the optimal virus inoculation dose and timepoint for initiation of antiviral treatment.

Antiviral studies in mice

Mice (anaesthetized using a mixture of 1.6 mg/mL zolazepam, 1.6 mg/mL tiletamine and 1.9 mg/mL xylazine) were infected intranasally with a sublethal dose of influenza virus as follows: 100 $TCID_{50}$ for A/WSN/33, A/Hong Kong/8/68 and A/WSN/33-NA/H274Y infection; 400 $TCID_{50}$ for B/Hong Kong/5/72 infection; and 4300 $TCID_{50}$ for A/Osaka/129/2009 infection.

Five days after inoculation, A/WSN/33-infected mice were treated with baloxavir marboxil [0.5, 1.5, 5, 15 or 50 mg/kg orally q12h; 10/group (Figure 3), and 5 mg/kg orally q12h; 15/group (Figure 2)], oseltamivir phosphate (5 or 50 eq mg/kg orally q12h; 10/group),[27] laninamivir octanoate (1 or 3 mg/kg intranasally q24h; 15/group),[28] zanamivir hydrate (10 mg/kg intranasally q12h; 15/group),[29] favipiravir (50 or 150 mg/kg orally q12h; 15/group)[30] or vehicle [0.5% (w/v) methyl cellulose in aqueous solution; 15/group (Figure 2) and 10/group (Figure 3)], for 1 day.

Mice infected with other types/subtypes of influenza viruses were treated at day 5 post-infection with baloxavir marboxil (0.5, 1.5, 5, 15 or 50 mg/kg, q12h, for A/Hong Kong/8/68, B/Hong Kong/5/72 and A/WSN/33-NA/H274Y infection, and 0.5 or 5 mg/kg, q12h, for A/Osaka/129/2009 infection), oseltamivir phosphate (5 or 50 eq mg/kg, q12h, for A/Hong Kong/8/68 and B/Hong Kong/5/72 infection, and 5 eq mg/kg, q12h, for A/WSN/33-NA/H274Y and A/Osaka/129/2009 infection) or vehicle, for 1 day. Five A/Osaka/129/2009-, 10 A/Hong Kong/8/68-, 10 A/WSN/33-NA/H274Y- or 15 B/Hong Kong/5/72-infected mice were included per dose group.

To investigate the PD effects of baloxavir marboxil on viral load reduction, A/WSN/33-infected mice were treated at day 5 post-infection with subcutaneous (back of the neck) baloxavir acid as follows: 0.25, 0.5, 1, 2, 4 or 8 mg/kg, q24h; 0.125, 0.25, 0.5, 1, 2 or 4 mg/kg, q12h; and 0.0625, 0.125, 0.25, 0.5, 1 or 2 mg/kg q6h (five per group), for 1 day.

After 24 h, mice were euthanized by cervical dislocation and virus titres in lung homogenates were determined. The $\Delta\log_{10}$ $TCID_{50}$/mL value for each mouse was calculated by subtracting the mean virus titre in vehicle-treated mice from individual virus titres in the substance-treated mouse.

PK in infected mice

A/WSN/33-infected mice were treated orally with baloxavir marboxil (0.5, 1.5, 5, 15 or 50 mg/kg) and blood was taken at 0.5, 1, 2, 4, 6, 8, 10 or 12 h after dosing (three mice per group per timepoint). Another group of A/WSN/33-infected mice received subcutaneous (back of the neck) baloxavir acid (0.125, 0.5, 2 or 8 mg/kg) and blood was taken at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 or 24 h after dosing at 0.125, 0.5 or 2 mg/kg or at 0.083, 0.333, 0.5, 1, 2, 4, 6, 8 or 24 h after dosing at 8 mg/kg (three mice per group per timepoint).

Plasma concentrations of baloxavir acid and baloxavir marboxil were determined by LC–tandem MS. Plasma concentration data following single dosing for 0.125, 0.5, 2 and 8 mg/kg doses were averaged by dose and nominal time. Mean plasma concentrations at each sampling time and each dose level were used for the PK analysis. For oral administration of baloxavir marboxil, C_{max}, T_{max}, terminal elimination half-life ($t_{1/2,z}$), AUC_{0-12} and $AUC_{0-\infty}$ were calculated by WinNonlin Version 6.2.1 (Certara USA Inc., Princeton, NJ, USA) based on a non-compartment model with uniform weighting. For subcutaneous baloxavir acid administration, C_{max}, plasma concentration at 24 h after initial dosing (C_{24}), plasma concentration at the end of the dosing interval (C_τ), AUC_{0-24} and time to reach plasma concentration of 2, 10 or 50 ng/mL ($T_{>2}$, $T_{>10}$ or $T_{>50}$, respectively) were estimated in the same manner. The same PK parameters for 0.0625, 0.25, 1 and 4 mg/kg doses (which were not administered to infected mice for PK evaluation) were mathematically scaled by extrapolation or interpolation based on the PK parameters in infected mice dosed at 0.125, 0.5, 2 and 8 mg/kg. Scaling was conducted as follows: each parameter's value at the 0.0625 mg/kg dose was calculated as 1/2× the parameter's value at the 0.125 mg/kg dose; similarly, values at the 0.25 mg/kg dose were calculated as 2/3× the value at the 0.125 mg/kg dose + 1/3× the value at the 0.5 mg/kg dose, values at 1 mg/kg were calculated as 2/3× the value at 0.5 mg/kg + 1/3× the value at 2 mg/kg and values at 4 mg/kg were calculated as 2/3× the value at 2 mg/kg + 1/3× the value at 8 mg/kg.

PK/PD analysis

To investigate the relationship between antiviral activity and PK parameters following subcutaneous baloxavir acid dosing in the A/WSN/33 infection model, the sigmoid E_{max} model was applied to virus titre data derived from individual mice in the PD studies, and to the PK parameters for doses 0.125, 0.5, 2 and 8 mg/kg that were calculated from the observed mean plasma concentrations at each dose and timepoint, and to the PK parameters for 0.0625, 0.25, 1 and 4 mg/kg doses that were mathematically scaled in PK studies as described above. The dependent variable (y) was the virus titre (log_{10} $TCID_{50}$/mL) at 24 h after initial dosing and the independent variable (x) was each PK parameter: $y = E_0 - E_{max} \times x^\gamma/(EC_{50}{}^\gamma + x^\gamma)$, where E_0 is the baseline effect, E_{max} is the maximum effect, EC_{50} reflects 50% of the maximum effect and γ is a slope parameter. The linear model $y = E_0 - \beta x$ was also applied, where β is a regression coefficient. In this case, AUC_{0-24}, C_{max}, C_{24} and C_τ were used as the logarithmic values and the other PK parameters as the anti-logarithm. Model fitness was evaluated by the coefficient of determination R^2 adjusted for degrees of freedom.

Statistical analyses

Differences in lung virus titres 24 h after initial dosing of baloxavir marboxil, other anti-influenza drugs (oseltamivir phosphate 5 eq mg/kg q12h, zanamivir hydrate 10 mg/kg q12h, laninamivir octanoate 1 mg/kg q24h and favipiravir 50 mg/kg q12h) and vehicle were analysed by pairwise comparison with the fixed-sequence procedure for multiple testing under a one-way analysis of variance (ANOVA) model. Exploratory comparison of virus titres with baloxavir marboxil, other anti-influenza drugs (oseltamivir phosphate 50 eq mg/kg q12h, laninamivir octanoate 3 mg/kg q24h and favipiravir 150 mg/kg q12h) and vehicle was performed using a one-way ANOVA model without multiplicity adjustment. To analyse differences in viral load reduction, Dunnett's test was applied for comparing the differences in mean log_{10} $TCID_{50}$/mL values between baloxavir marboxil- and oseltamivir phosphate-treated groups.

Statistical analyses were performed using SAS version 9.2 for Windows (SAS Institute, Cary, NC, USA). Two-sided adjusted P values below 0.05 were considered statistically significant.

Results

Murine model of influenza virus infection

A sub-lethal murine model was established by measuring virus titres in the lungs of mice inoculated with A/WSN/33 at 0.25×, 0.5× or 1× LD_{50}. In all groups, virus titres reached a mean ± SD of 6.03 ± 0.35 to 6.55 ± 0.22 log_{10} $TCID_{50}$/mL by day 2 post-infection. Virus titres remained at similar levels to day 5 and then declined (Figure 1). Up to day 5, mice receiving the 0.25× LD_{50} infection dose did not show significant body weight loss (Figure S1, available as Supplementary data at *JAC* Online). Furthermore, all these mice survived at 14 days post-infection. Therefore, we selected the virus inoculation dose of 0.25× LD_{50} (100 $TCID_{50}$) on day 0 for subsequent investigations, initiated the treatment with test substances on day 5 post-infection and evaluated virus titre reduction on day 6 post-infection.

Antiviral activities of oral baloxavir marboxil in the murine model

All antivirals tested in A/WSN/33-infected mice showed significant reductions in mean viral load compared with vehicle. Lung virus titres in mice receiving baloxavir marboxil 5 mg/kg, q12h, were significantly lower than for each NAI or favipiravir (Figures 2 and 3). The effect of baloxavir marboxil was dose dependent (Figure 3). Compared with the clinically equivalent or higher dose of oseltamivir phosphate, baloxavir marboxil ≥1.5 mg/kg, q12h, significantly reduced virus titres. In mice receiving baloxavir marboxil ≥15 mg/kg, q12h, virus titres declined by >3 log_{10} $TCID_{50}$/mL compared with vehicle and by 2 log_{10} $TCID_{50}$/mL compared with oseltamivir phosphate 5 or 50 eq mg/kg, q12h.

In mice infected with other influenza virus types/subtypes, including an NAI-resistant variant and the clinical H1N1pdm09 strain, virus titres were significantly lower with baloxavir marboxil 5 mg/kg, q12h, than with vehicle (data not shown), and treatment with baloxavir marboxil 15 mg/kg, q12h, exhibited a ≥100-fold influenza A virus titre reduction or ≥10-fold influenza B virus titre reduction compared with the clinically equivalent or

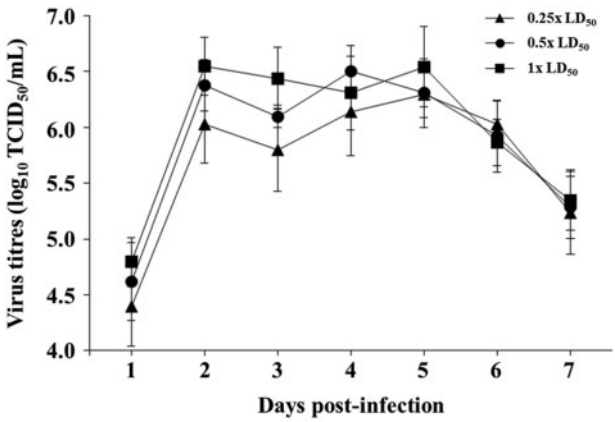

Figure 1. Viral growth curves in mice infected with A(H1N1) influenza virus. Mice were infected with the A/WSN/33 (H1N1) strain at 0.25× (triangle), 0.5× (circle) or 1× (square) LD_{50} and virus titres in their lungs were measured at each timepoint. Data represent the mean ± SD of five mice.

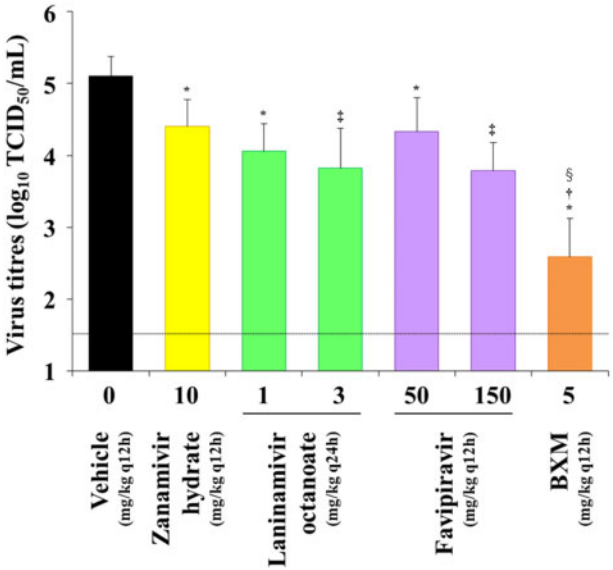

Figure 2. Comparative *in vivo* efficacy of baloxavir marboxil (BXM; 5 mg/kg, q12h) and other anti-influenza drugs in mice infected with 0.25× LD_{50} (100 $TCID_{50}$) of the A/WSN/33 strain. Virus titres in lungs 24 h after initial antiviral dosing (6 days after influenza infection). Each bar represents the mean ± SD of 15 mice. *Adjusted $P < 0.0001$ versus vehicle; †adjusted $P < 0.0001$ versus zanamivir hydrate 10 mg/kg, q12h, laninamivir octanoate 1 mg/kg, q24h and favipiravir 50 mg/kg, q12h (the pairwise comparisons with the fixed-sequence procedure for multiple testing by using a one-way ANOVA model); ‡$P < 0.0001$ versus vehicle; and §$P < 0.0001$ versus laninamivir octanoate 3 mg/kg, q24h, and favipiravir 150 mg/kg, q12h (the pairwise comparisons by using a one-way ANOVA model).

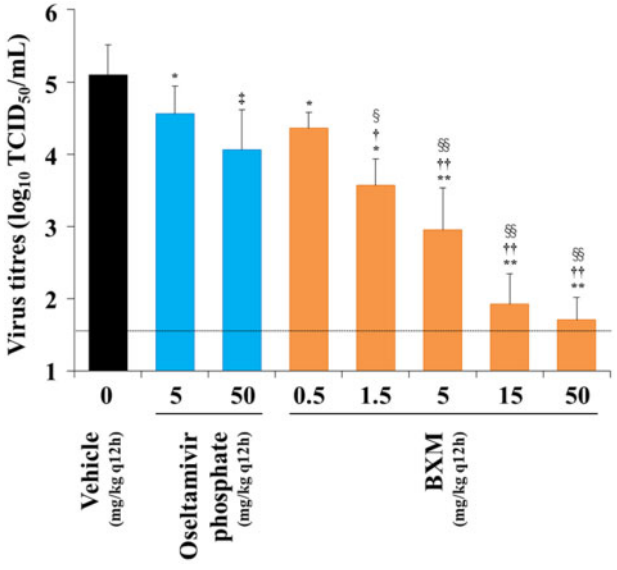

Figure 3. Dose dependency of virus inhibition after dosing with baloxavir marboxil (BXM; 0.5–50 mg/kg, q12h), oseltamivir phosphate [5 or 50 eq mg/kg, q12h (corresponding to therapeutic and supratherapeutic doses)] or vehicle in mice infected with 0.25× LD_{50} (100 $TCID_{50}$) of the A/WSN/33 strain. Virus titres in lungs 24 h after initial antiviral dosing (6 days after influenza infection). Each bar represents the mean ± SD of 10 mice. *Adjusted $P < 0.01$ and **adjusted $P < 0.0001$ versus vehicle; †adjusted $P < 0.01$ and ††adjusted $P < 0.0001$ versus oseltamivir phosphate 5 eq mg/kg, q12h (the pairwise comparisons with the fixed-sequence procedure by using a one-way ANOVA model); ‡$P < 0.0001$ versus vehicle; and §$P < 0.05$ and §§$P < 0.0001$ versus oseltamivir phosphate 50 eq mg/kg, q12h (the pairwise comparisons by using a one-way ANOVA model).

higher dose of oseltamivir phosphate (Figure 4). Of note, treatment with baloxavir marboxil 5 mg/kg, q12h, was sufficient to lead to ≥100-fold virus titre reduction in mice infected with the H1N1pdm09 strain.

PK of baloxavir acid after oral baloxavir marboxil administration

To further assess the dose-dependent efficacy of baloxavir marboxil, we measured plasma concentrations of baloxavir acid in A/WSN/33-infected mice. Baloxavir acid plasma exposure increased dose proportionally up to 15 mg/kg of oral baloxavir marboxil, whereas exposure after treatment with oral baloxavir marboxil 50 mg/kg was lower than expected (Figure 5 and Table S1). Additionally, C_{max}, AUC_{0-12} and $AUC_{0-\infty}$ increased dose proportionally between 0.5 and 15 mg/kg doses of baloxavir marboxil, whereas with 50 mg/kg these values were lower than expected (Table 1). Furthermore, C_{max} was reached at 0.5–2 h post-administration of baloxavir marboxil; plasma concentrations of baloxavir acid then declined with a $t_{1/2,z}$ of 2.24–3.14 h. T_{max} following a baloxavir marboxil dose of 50 mg/kg was slightly longer than for other doses (Table 1). Baloxavir marboxil was undetectable in almost all infected mice (data not shown), suggesting rapid conversion into baloxavir acid.

PK of baloxavir acid after subcutaneous administration of baloxavir acid

Because linearity of baloxavir acid plasma exposure was not observed at higher doses of oral baloxavir marboxil (15–50 mg/kg), we evaluated subcutaneous administration of baloxavir acid at 0.125, 0.5, 2 and 8 mg/kg. Baloxavir acid plasma concentration increased dose proportionally from 0.125 to 8 mg/kg and with the 8 mg/kg dose was similar to or higher than that following oral administration of baloxavir marboxil 50 mg/kg. This suggests that subcutaneous administration of baloxavir acid solution was superior to oral baloxavir marboxil administration in mice in terms of dose-proportional increase in plasma baloxavir acid (Figure 6).

Antiviral activities of subcutaneous baloxavir acid in the lungs of infected mice

Following subcutaneous administration of baloxavir acid solution at doses between 0.0625 and 8 mg/kg q24h, q12h or q6h in A/WSN/33-infected mice, virus titres in all baloxavir acid-treated groups at 24 h post-treatment initiation were numerically lower than in groups treated with vehicle or oseltamivir phosphate 5 eq mg/kg, q12h (Table S2).

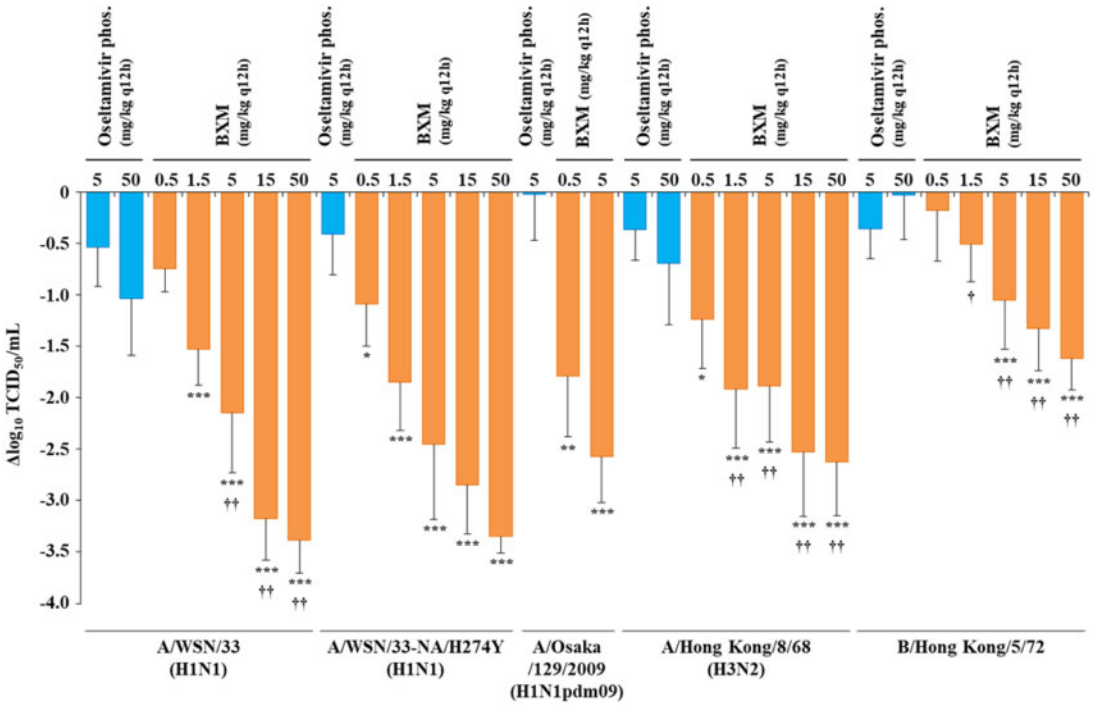

Figure 4. Efficacy of baloxavir marboxil (BXM; 0.5–50 mg/kg, q12h) or oseltamivir phosphate (5 or 50 eq mg/kg) against multiple strains of influenza virus. Virus titres in lungs 24 h after initial antiviral dosing (6 days after influenza infection). Each bar represents the mean ± SD difference from the mean of $\log_{10}$ $TCID_{50}$/mL in the vehicle group in 10 mice except for the experiment for A/Osaka/129/2009 (5 mice) and B/Hong Kong/5/72 (15 mice). Virus titres of the vehicle groups were as follows: A/WSN/33, 5.10±0.41 $\log_{10}$ $TCID_{50}$/mL; A/WSN/33-NA/H274Y, 4.95±0.41 $\log_{10}$ $TCID_{50}$/mL; A/Osaka/129/2009, 5.41±0.71 $\log_{10}$ $TCID_{50}$/mL; A/Hong Kong/8/68, 4.97±0.38 $\log_{10}$ $TCID_{50}$/mL; and B/Hong Kong/5/72, 3.92±0.35 $\log_{10}$ $TCID_{50}$/mL. *Adjusted $P < 0.01$, **adjusted $P < 0.001$ and ***adjusted $P < 0.0001$ versus oseltamivir phosphate 5 eq mg/kg, q12h; and †adjusted $P < 0.05$ and ††adjusted $P < 0.0001$ versus oseltamivir phosphate 50 eq mg/kg, q12h (Dunnett's test).

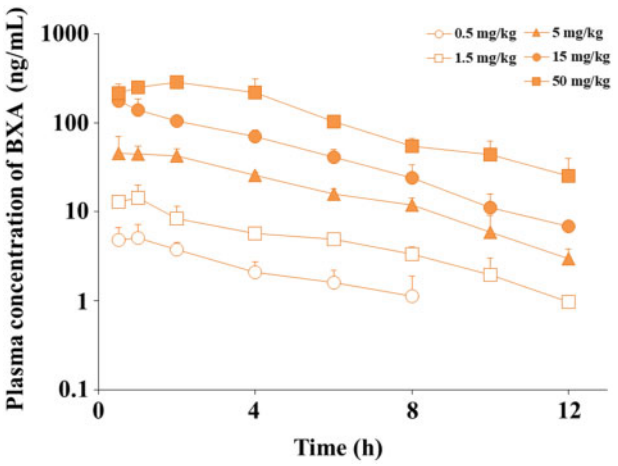

Figure 5. Plasma concentration of baloxavir acid (BXA) in infected mice following oral treatment with baloxavir marboxil at 0.5–50 mg/kg. The plasma concentration of BXA in A/WSN/33 (H1N1) strain-infected mice was measured at each timepoint. Data represent the mean ± SD of three mice. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

PK/PD analysis of baloxavir acid in infected mice

PK parameters for 0.125, 0.5, 2 and 8 mg/kg doses of subcutaneous baloxavir acid were calculated from the observed mean plasma concentrations at each dose and time, and 0.0625, 0.25, 1 and 4 mg/kg doses were mathematically scaled (Table S3), and correlation with the antiviral activity of baloxavir acid was assessed. First, the sigmoid E_{max} model was applied (Figure S2). The adjusted R^2 of AUC_{0-24}, C_{max}, C_{24}, C_τ and $T_{>50}$ was 0.395, 0.236, 0.581, 0.581 and 0.285, respectively, and was not calculated for $T_{>2}$ and $T_{>10}$ because model convergence was not achieved; the adjusted R^2 values of C_{24} and C_τ were therefore the largest among the PK parameters (Table 2). The linear model was then applied (Figure 7). The adjusted R^2 of AUC_{0-24}, C_{max}, C_{24}, C_τ, $T_{>2}$, $T_{>10}$ and $T_{>50}$ was 0.392, 0.241, 0.522, 0.527, 0.317, 0.508 and

Table 1. PK parameters of baloxavir acid after oral administration of baloxavir marboxil in A/WSN/33 (H1N1) strain-infected mice

	Baloxavir marboxil dose (mg/kg)				
	0.5	1.5	5	15	50
C_{max} (ng/mL)	5.05	14.3	45.7	175	284
T_{max} (h)	1.00	1.00	0.500	0.500	2.00
$t_{1/2,z}$ (h)[a]	2.24	2.56	2.45	2.26	3.14
$AUC_{0-\infty}$ (ng·h/mL)	22.7	66.1	252	670	1690
AUC_{0-12} (ng·h/mL)	22.0	62.6	242	648	1580

[a]0.5 mg/kg, $t_{1/2,4-10\ h}$; 1.5–50 mg/kg, $t_{1/2,6-12\ h}$.

0.295, respectively. C_τ again exhibited the largest adjusted R^2 (Table 3). These results indicate that C_τ or C_{24} correlated more strongly than did other PK parameters with virus titre reduction at 24 h after dosing with baloxavir acid in the murine infection model.

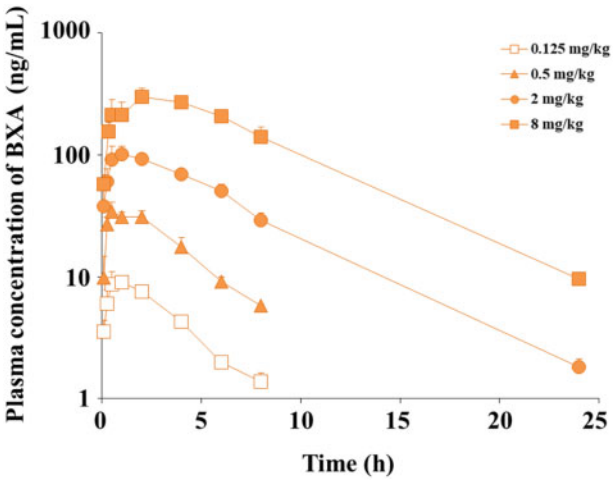

Figure 6. Plasma concentration of baloxavir acid (BXA) in infected mice following subcutaneous administration of BXA at 0.125–8 mg/kg. The plasma concentration of BXA in A/WSN/33 (H1N1) strain-infected mice was measured at each timepoint. Data represent the mean ± SD of three mice. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Discussion

In this study, we first determined the murine model of influenza virus infection for investigation of the *in vivo* efficacy and PK/PD profile of baloxavir acid. Although viral load reduction is a direct virological endpoint for patients treated with a viral replication inhibitor, this endpoint has not been previously applied to any PK/PD study of NAIs because the range of virus titre reduction was insufficient for precise PK/PD correlation, even when using suprathera-peutic doses. Therefore, PK/PD analyses of NAIs have previously been based on survival rates of mice or clinical symptoms of infected ferrets.[14–16] Here, we focused on the virological endpoint of rapid reduction of virus burden following baloxavir marboxil treatment; therefore, comparisons with PK/PD studies of NAI are limited. We used a sub-lethal murine model, in which treatment was initiated 5 days after virus inoculation followed by lung virus titre quantification at 24 h post-dosing. Virus titre at 5 days post-infection (6.29 ± 0.21 $\log_{10}$ $TCID_{50}$/mL) in this model, just before the decline in virus titre, is similar to the baseline virus titre observed in otherwise healthy influenza patients (viral loads ranged from 5.56 ± 1.89 to 5.94 ± 1.69 $\log_{10}$ $TCID_{50}$/mL).[25] We demonstrated that replication of influenza A(H1N1), A(H1N1)pdm09, A(H3N2), type B or NAI-resistant A(H1N1) virus was more strongly suppressed by baloxavir marboxil at the dose of 5 mg/kg, q12h, than by oseltamivir phosphate, although a slightly different antiviral activity of baloxavir marboxil between type A and B was observed in this study and in our previous work.[20,21] The inhibitory values for type A virus strains were 4- to 5-fold more potent in the yield-reduction assay and 6- to 12-fold more potent in

Table 2. Analysis of PK parameters of baloxavir acid in the sigmoid E_{max} model

PK parameter	Model parameter	Estimate	Standard error	95% CI	*P*	R^2 (adjusted R^2)
AUC_{0-24} (ng·h/mL)	E_0	3.821	0.116	3.590, 4.052	<0.0001	0.415
	E_{max}	1.091	0.170	0.752, 1.429	<0.0001	(0.395)
	EC_{50}	431.596	81.757	269.068, 594.124	<0.0001	
	γ	4.170	2.291	−0.385, 8.725	0.0722	
C_{max} (ng/mL)	E_0	3.806	0.208	3.391, 4.220	<0.0001	0.261
	E_{max}	1.022	0.333	0.360, 1.683	0.0029	(0.236)
	EC_{50}	42.528	14.034	14.629, 70.427	0.0032	
	γ	2.421	1.972	−1.500, 6.342	0.2230	
C_{24} (ng/mL)	E_0	3.928	0.116	3.697, 4.159	<0.0001	0.595
	E_{max}	1.499	0.206	1.090, 1.908	<0.0001	(0.581)
	EC_{50}	4.767	1.047	2.687, 6.848	<0.0001	
	γ	1.843	0.649	0.554, 3.133	0.0056	
C_τ (ng/mL)	E_0	3.958	0.126	3.707, 4.210	<0.0001	0.595
	E_{max}	1.554	0.233	1.091, 2.016	<0.0001	(0.581)
	EC_{50}	4.108	0.961	2.199, 6.018	<0.0001	
	γ	1.697	0.604	0.498, 2.897	0.0061	
$T_{>50}$ (h)	E_0	3.588	0.094	3.403, 3.774	<0.0001	0.309
	E_{max}	1.913	2.859	−3.770, 7.596	0.5051	(0.285)
	EC_{50}	16.534	38.174	−59.353, 92.421	0.6660	
	γ	1.324	1.438	−1.534, 4.183	0.3597	

Adjusted R^2 is the coefficient of determination R^2 adjusted for degrees of freedom; *P* value <0.05 considered statistically significant.

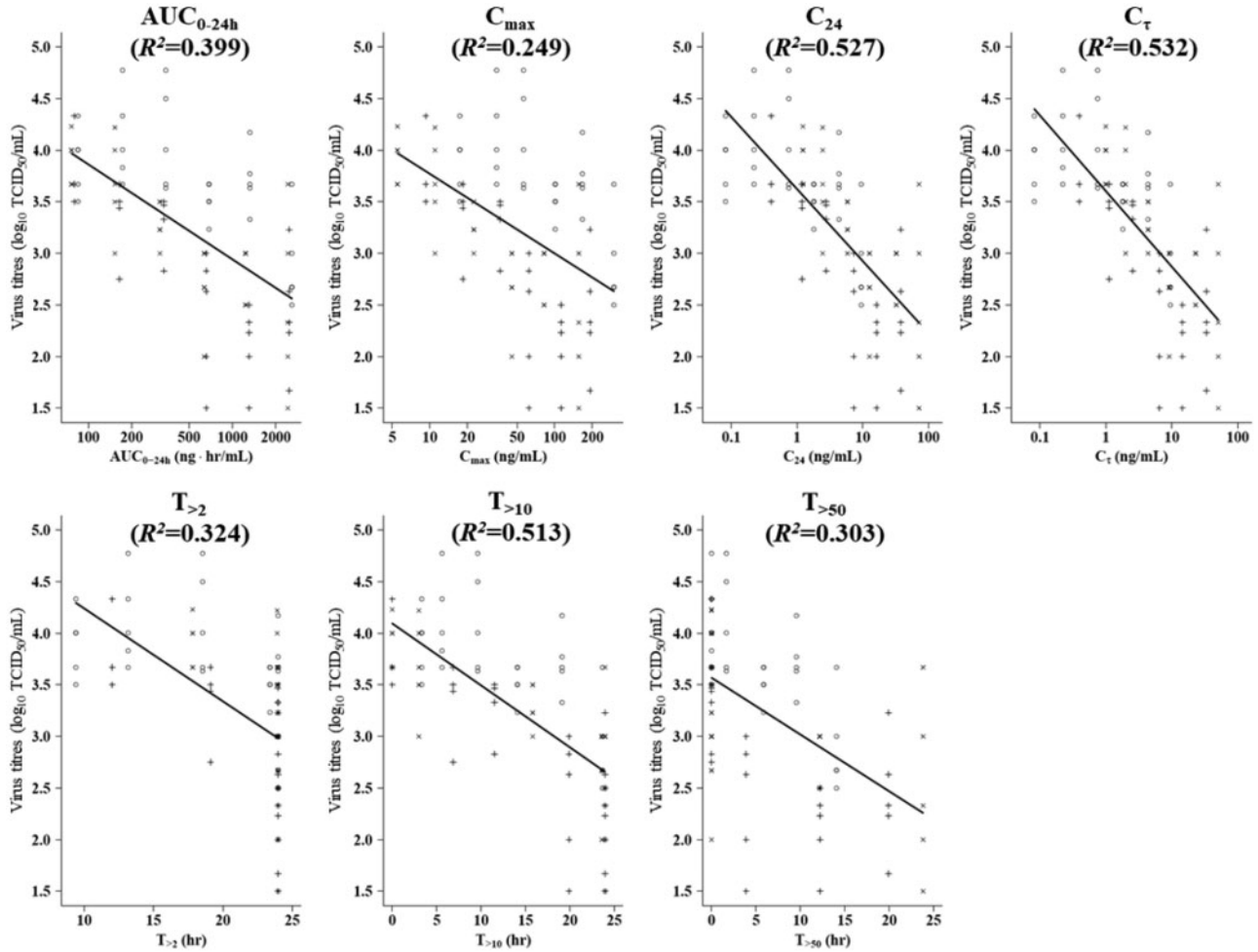

Figure 7. Estimated linear curves between each PK parameter and virus titres in the lungs. The linear model was applied to evaluate the relationship between each PK parameter and virus titres; 17 data points overlapped other data points because the same viral titre was observed in different mice (virus titres by animal are summarized in Table S4). Each symbol represents an observed value: circles, q24h; plus symbols, q12h; and crosses, q6h. Solid lines represent estimated curves.

the plaque-reduction assay than values for type B virus strains. Baloxavir marboxil reduces the virus titre in mice *in vivo* according to the types of influenza virus, similar to the *in vitro* activity of baloxavir acid. These results validate the use of virus titre changes in the PK/PD analysis of baloxavir marboxil/baloxavir acid.

Consistent with the dose-proportional increase in plasma concentration and the PK parameters of baloxavir acid following oral baloxavir marboxil treatment, dose-dependent reduction in virus titres in the lungs of mice receiving baloxavir marboxil was observed. However, the dose-proportional increase in plasma baloxavir acid concentration was limited to baloxavir marboxil doses of 0.5–15 mg/kg, suggesting that the linearity of plasma exposure to baloxavir acid would not be exhibited at higher doses of oral baloxavir marboxil. Conversely, subcutaneous administration of baloxavir acid showed a dose-proportional increase in baloxavir acid exposure even at higher baloxavir acid doses; therefore, we applied subcutaneous baloxavir acid treatment for the PK/PD relationship analysis.

Our PK/PD analysis of PK parameters and virus titre reduction showed that C_τ best predicted the antiviral activity of baloxavir acid. A limitation of our PD analysis is that it only assessed the A/WSN/33 strain. Although C_τ was the best PK parameter predicting the virus titres, we presume that C_τ is different for other influenza strains. Therefore, the target C_τ was not defined from the EC_{50} derived from the linear and sigmoidal models. As shown in Figure 4, the oral administration of baloxavir marboxil at 15 mg/kg twice daily decreased the virus titre in mice infected with A/WSN/33 (H1N1), A/Hong Kong/8/68 (H3N2) and B/Hong Kong/5/72 strains by 2.64, 2.16 and 0.97 $\log_{10}$ $TCID_{50}$/mL, respectively, compared with oseltamivir phosphate at 5 eq mg/kg. In order to provide superior reduction of virus titres compared with oseltamivir, we explored a 1 log reduction compared with oseltamivir phosphate for both influenza A and B viruses; therefore, we defined the target C_τ value of baloxavir acid as more than 6.85 ng/mL, which was the plasma concentration obtained with oral baloxavir marboxil at a dose of 15 mg/kg, q12h (Table S1). In a recent non-clinical study in nude mice, daily administration with a

Table 3. Analysis of PK parameters of baloxavir acid in the linear model

PK parameter	Model parameter	Estimate	Standard error	95% CI	*P*	R^2 (adjusted R^2)
AUC_{0-24} (ng·h/mL)	E_0	5.705	0.326	5.056, 6.354	<0.0001	0.399
	β	0.400	0.052	0.296, 0.504	<0.0001	(0.392)
C_{max} (ng/mL)	E_0	4.542	0.248	4.050, 5.034	<0.0001	0.249
	β	0.335	0.062	0.212, 0.458	<0.0001	(0.241)
C_{24} (ng/mL)	E_0	3.624	0.066	3.493, 3.756	<0.0001	0.527
	β	0.303	0.031	0.242, 0.364	<0.0001	(0.522)
C_τ (ng/mL)	E_0	3.603	0.064	3.475, 3.731	<0.0001	0.532
	β	0.318	0.032	0.255, 0.381	<0.0001	(0.527)
$T_{>2}$ (h)	E_0	5.141	0.297	4.550, 5.732	<0.0001	0.324
	β	0.090	0.014	0.062, 0.117	<0.0001	(0.317)
$T_{>10}$ (h)	E_0	4.095	0.103	3.890, 4.301	<0.0001	0.513
	β	0.060	0.006	0.048, 0.072	<0.0001	(0.508)
$T_{>50}$ (h)	E_0	3.569	0.083	3.403, 3.734	<0.0001	0.303
	β	0.055	0.009	0.037, 0.072	<0.0001	(0.295)

Adjusted R^2 is the coefficient of determination R^2 adjusted for degree of freedom; *P* value <0.05 is considered statistically significant.

suboptimal dose of baloxavir marboxil (10 mg/kg, q24h) did not clear the virus from the respiratory organs.[31] A possible reason for the lower antiviral activity may be a longer interval of drug administration (q24h) because the half-life of baloxavir acid in mouse plasma ranges from 2.24 to 3.14 h. As shown with multiple-dosing treatment,[22–24] q12h administration raises C_τ for baloxavir acid and exhibits potent and sustained antiviral activity. These results support our findings that it is important to maintain an appropriate baloxavir acid concentration in mouse plasma to maximize the antiviral efficacy. In clinical trials, human PK data showed that single-dose oral baloxavir marboxil treatment resulted in a >10-fold longer half-life of baloxavir acid in plasma compared with that in mice and plasma concentrations of baloxavir acid exceeded and maintained the target plasma concentration for longer than 120 h.[19,32] Phase 3 baloxavir marboxil studies clearly demonstrated that patients with uncomplicated influenza, including adults and adolescents, exhibited greater viral load reduction 24 h after single-dose oral baloxavir marboxil treatment than after placebo (3.5 $\log_{10}$ $TCID_{50}$/mL reduction) or treatment with oseltamivir phosphate (2.0 $\log_{10}$ $TCID_{50}$/mL reduction) and the significant antiviral activity of baloxavir marboxil was sustained for several days. A different antiviral response to baloxavir marboxil by type A and type B viruses was also observed in other clinical studies.[33,34] We therefore believe that our mouse model replicates closely the influenza viral kinetics seen in humans. The model may have utility in future studies for evaluating residual virus burden following antiviral treatment and thus the potential for transmission.[35] Symptom resolution was faster with baloxavir marboxil than placebo, but not oseltamivir phosphate, in type A virus infections.[25,34] These clinical findings suggest that our PK/PD analysis based on virus titre reduction in the murine model could be useful in predicting potent viral load reduction in patients, but might be limited in predicting a shorter time to symptom resolution in otherwise healthy influenza patients. Treatment with antiviral agents may fail to directly reflect the symptom alleviation benefit for such patients because influenza illness is closely linked to host immune response. Differences in host responses between humans and mice might be considered in both virological and therapeutic endpoints in future non-clinical studies. A limitation of our study is that it is not feasible to take sequential blood samples from the same individual mice; therefore, PK parameters must be obtained from groups of animals for different doses to be applied in PK/PD analysis. Another limitation is the large difference in PK of baloxavir acid in plasma between humans and mice. Additionally, the effects of possible interactions between baloxavir acid and plasma proteins were not considered in our study. Regimens in murine models mimicking the human PK of baloxavir acid are warranted for further assessment of baloxavir marboxil.

A previously reported PK/PD analysis based on *in vivo* virus titre reduction evaluated the use of a PB2 inhibitor in an apparently lethal influenza A virus infection model, with treatment administered 24 h post-infection when virus titres were still increasing.[18] In contrast, our murine model was designed for infection at sub-lethal doses where severe host response, such as significant body weight loss, was not observed and for initiation of antiviral treatment immediately before the decline in virus titres. Indeed, none of the mice infected at 0.25× LD_{50} in our model died because of the infection and any associated weight loss was modest and rapidly reversible. In our model, oseltamivir phosphate showed 0.5–1 $\log_{10}$ reduction in virus titres compared with vehicle, consistent with the titre reduction observed in clinical studies in which virus titres were already decreasing at day 2 in otherwise healthy placebo-treated influenza patients.[25,36]

In summary, we demonstrated that oral treatment with baloxavir marboxil exhibited rapid and potent virus titre reduction regardless of type/subtype of seasonal influenza virus in the sub-lethal murine infection model. Furthermore, we showed that C_τ or C_{24} was the PK parameter that predicted the antiviral activity of baloxavir acid. To the best of our knowledge, this is the first report on influenza antivirals to describe the PK/PD correlation based on reduction in lung virus titres in a sub-lethal murine influenza model. Our findings suggest that this PK/PD model could be helpful in predicting and maximizing virological outcomes in clinical settings of baloxavir marboxil.

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Supplementary data

Figures S1 and S2 and Tables S1 to S4 are available as Supplementary data at *JAC* Online.

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