Studies on the efficacy of anti-influenza drug baloxavir marboxil against animal-derived influenza viruses

（動物由来インフルエンザウイルスに対する
抗インフルエンザ薬バロキサビルマルボキシルの
有効性に関する研究）

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Contents

Abbreviations

A20T:	alanine-to-threonine at amino acid position 20
A37S:	alanine-to-serine at amino acid position 37
A37T:	alanine-to-threonine at amino acid position 37
A549:	human lung adenocarcinoma epithelial
A85T:	alanine-to-threonine at amino acid position 85
ARDS:	acute respiratory distress syndrome
bid:	bis in die
BXA:	baloxavir acid
BXM:	baloxavir marboxil
C_{24}:	target plasma concentration 24 hours after a single-dose
C_{120}:	target plasma concentration 120 hours after a single-dose
CEN:	cap-dependent endonuclease
CI:	combination index
CPE:	cytopathic effect
dpi:	days post-infection
E23G:	glutamic acid-to-glycine at amino acid position 23
EC_{50}:	50% effective concentration
EC_{90}:	90% effective concentration
FBS:	fetal bovine serum
FPV:	favipiravir
GISAID:	Global Initiative on Sharing All Influenza Data
H275Y:	histidine-to-tyrosine at amino acid position 275
HE:	hematoxylin and eosin
HPAIV(s):	highly pathogenic avian influenza virus(es)
I127V:	isoleucine-to-valine at amino acid position 127
I38F:	isoleucine-to-phenylalanine at amino acid position 38
I38L:	isoleucine-to-leucine at amino acid position 38
I38M:	isoleucine-to-methionine at amino acid position 38
I38T:	isoleucine-to-threonine at amino acid position 38
IAV(s):	influenza A virus(es)
IL:	interleukin
M61I:	methionine-to-isoleucine at amino acid position 61
MCP:	monocyte chemoattractant protein
MDCK:	Madin-Darby canine kidney

MEM:	minimum essential medium
MLD_{50}:	50% mouse lethal dose
MTT:	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NA:	neuraminidase
N294S:	asparagine-to-serine at amino acid position 294
NAIs:	neuraminidase inhibitor(s)
NCBI:	National Center for Biotechnology Information
OSA:	oseltamivir acid
OSP:	oseltamivir phosphate
PA:	polymerase acidic
PA/I38:	amino acid position 38 in the PA
PRV:	peramivir trihydrate
R292K:	arginine-to-lysine at amino acid position 292
RNA:	ribonucleic acid
RT-qPCR:	quantitative real-time reverse-transcriptase polymerase chain reaction
SD:	standard deviation
$TCID_{50}$:	50% tissue culture infectious dose
WT:	wild-type
Y24H:	tyrosine-to-histidine at amino acid position 24

Notes

Contents of the present thesis were published in the following articles.

1. **Taniguchi K, Ando Y, Nobori H, Toba S, Noshi T, Kobayashi M, Kawai M, Yoshida R, Sato A, Shishido T, Naito A, Matsuno K, Okamatsu M, Sakoda Y, Kida H.** Inhibition of avian-origin influenza A(H7N9) virus by the novel cap-dependent endonuclease inhibitor baloxavir marboxil. Scientific Reports, 9:3466, 2019.

2. **Taniguchi K, Ando Y, Kobayashi M, Toba S, Nobori H, Sanaki T, Noshi T, Kawai M, Yoshida R, Sato A, Shishido T, Naito A, Matsuno K, Okamatsu M, Sakoda Y, Kida H.** Characterization of the *in vitro* and *in vivo* efficacy of baloxavir marboxil against H5 highly pathogenic avian influenza virus infection. Viruses, 14:111, 2022.

3. **Taniguchi K, Noshi T, Omoto S, Sato A, Shishido T, Matsuno K, Okamatsu M, Scott Krauss, Richard J Webby, Sakoda Y, Kida H.** The impact of PA/I38 substitution and PA polymorphisms on susceptibility of zoonotic influenza A viruses to baloxavir. Archives of Virology, in press.

General introduction

Influenza is a respiratory infection in humans and animals caused by influenza viruses. Since the 20th century, influenza pandemics have occurred many times. One of the more striking evolutionary features of influenza A viruses (IAVs) is genetic reassortment, which leads to the emergence of pandemic-causing viruses (1, 2). IAVs maintained in aquatic wild birds undergo genetic reassortment *via* poultry and livestock, facilitating the emergence of novel viruses that can infect humans (3). Direct transmission of IAVs from animals to humans also poses a significant threat to human health due to potentially high morbidity and mortality (4, 5). Since human infection cases with high mortality and morbidity caused by A(H5N1) highly pathogenic avian influenza viruses (HPAIVs) were reported in 1997, cases of various subtypes of avian influenza viruses, such as A(H7N9) viruses in 2013, infecting humans have been sporadically reported (4-6). It is possible that all subtypes of influenza viruses could infect humans in the future; therefore, preparedness with broad-spectrum antiviral drugs is important for pandemic prophylaxis and treatment.

Early administration of antiviral drugs is crucial for the treatment in case of zoonotic IAV infection (7). The U.S. Centers for Disease Control and Prevention recommends the use of neuraminidase inhibitors (NAIs), oseltamivir and zanamivir, as antiviral drugs for humans infected with avian influenza viruses (7-9). However, NAI-resistant mutants, which have been detected in seasonal influenza viruses (10, 11), have emerged after NAI treatment in some cases (12, 13). These mutations were also observed during animal surveillance studies of circulating avian influenza viruses and have been identified in avian and human isolates as well, including an isolate from a patient treated with oseltamivir (13-15). Hence, expanding treatment options is important.

Baloxavir marboxil (BXM), which is converted metabolically to its active form baloxavir acid (BXA), is an orally available cap-dependent endonuclease (CEN) inhibitor approved for clinical use in adults and adolescents worldwide (16). BXA selectively inhibits CEN activity carried by polymerase acidic (PA) protein of influenza A and B viruses and suppresses the transcriptional process of the viral genome (17, 18). The amino acid sequence of the PA protein is highly conserved regardless of influenza virus subtype (17); therefore, BXA exhibits broad antiviral activities against several subtypes of influenza viruses, including zoonotic strains *in vitro* (18, 19). Moreover, BXA exhibits inhibitory efficacy on viral replication at lower doses than other anti-influenza drugs (18, 19). However, BXA susceptibility of zoonotic strains harboring NAI resistance mutations and the inhibitory efficacy of BXM on zoonotic strains in animal models

remain unclear. Furthermore, the amino acid substitution associated with reduced susceptibility to BXA [e.g., isoleucine-to-threonine, -phenylalanine or -methionine at amino acid position 38 (I38T, I38F or I38M) in the PA (PA/I38) N-terminal domain] has been reported in clinical settings (17, 20-22). However, the characterization of PA/I38 variant in zoonotic strains has not been sufficiently performed, and the knowledge on PA amino acid polymorphism, which can be a resistance mutation marker of BXA, has also been limited (23, 24).

In this study, the *in vitro* and *in vivo* efficacy of BXM against several zoonotic influenza virus strains was verified. In Chapter I, the efficacy of BXM against avian-origin A(H7N9) and A(H5N1) viruses were evaluated. In Chapter II, the impact of PA/I38 substitution and PA amino acid polymorphisms on BXA susceptibility of temporally and geographically distinct zoonotic IAVs was evaluated.

Chapter I

Characterization of the *in vitro* and *in vivo* efficacy of baloxavir marboxil against avian-origin A(H7N9) and A(H5N1) virus infection

Introduction

Sporadic influenza outbreaks caused by various subtypes [e.g., A(H5N1), A(H7N9), A(H5N8) and A(H9N2)] have been reported over the last decades (4-6). In particular, human infections with avian-origin A(H7N9) or A(H5N1) influenza viruses represent a serious threat to global health (4, 25, 26). In 2013, the first outbreak in humans with an Asian lineage A(H7N9) virus was reported in China (27, 28). Since then, A(H7N9) viruses have caused several epidemic waves, and laboratory-confirmed human cases admitted to hospital with A(H7N9) virus exhibited high case fatality rates caused by lung inflammation [e.g., severe pneumonia or acute respiratory distress syndrome (ARDS)] (29). The highest number of human cases of A(H7N9) viruses occurred in China from October 2016 and highly pathogenic A(H7N9) viruses were clinically isolated in 2017 (26, 29). Although no cases of human infection have been reported since 2019, it is an avian influenza virus that should be closely monitored (30). Human infections caused by A(H5N1) or the other subtypes of H5 HPAIVs have been reported worldwide with high mortality and morbidity since 1997 (4, 6). Infection with these viruses has been reported to cause severe pneumonia, resulting in the death of most patients due to progressive respiratory failure with ARDS (31, 32). In a previous report, the reduction in viral titers in the lungs by anti-influenza drug treatments was positively correlated with both the suppression of proinflammatory cytokine production and disease severity in A(H5N1) virus-infected murine model, suggesting that a strong inhibition of viral replication can reduce lung dysfunction (33).

HPAIVs are susceptible to NAIs, and treatment with oseltamivir has been reported to be effective in patients with A(H7N9) or A(H5N1) HPAIV infections (34, 35). However, the emergence of drug-resistant mutants [e.g., histidine-to-tyrosine at amino acid position 275 (H275Y) or arginine-to-lysine at amino acid position 292 (R292K) in the NA at N2 numbering] has been detected after NAI treatment in patients with these virus strains (12, 36). These mutated viruses were also detected during animal surveillance studies of circulating A(H7N9) or A(H5N1) viruses (13-15). NAIs have also shown limited ability to reduce viral titers; therefore, there may be limited virologic or therapeutic advantages, especially in patients with severe influenza with high viral loads (37-39). Therefore, novel antivirals that overcome the current limitations for the treatment of A(H7N9) or A(H5N1) virus infection are urgently required to decrease both morbidity and mortality in infected subjects and to assist in the preparedness to help mitigate widespread transmission in possible future pandemics.

BXA exhibits broad antiviral activities against several subtypes of influenza

viruses, including zoonotic strains *in vitro* (18, 19). However, BXA susceptibility to H7 or H5 HPAIVs isolated during various seasons and those harboring NAI-resistant mutations remains unknown. Moreover, compared with other anti-influenza drugs, BXM showed greater and more rapid reductions in viral load after treatment regimen initiation in clinical settings and *in vivo* studies. Nevertheless, due to the sporadic presentation of clinical cases, clinical assessments in A(H7N9) or A(H5N1) virus-infected patients are difficult. Therefore, it is beneficial to assess the antiviral effect of BXM and its regimen using *in vivo* models. For treating severe influenza cases, combination therapy using antiviral drugs with different mechanisms of action provides theoretical benefits (40, 41). Furthermore, studies have shown that combination therapy with oseltamivir phosphate (OSP) and BXM or favipiravir (FPV) is effective in mice (42-44). However, there is insufficient therapeutic evidence for combination therapy with BXM and OSP in infected animal models of zoonotic strains.

In this chapter, the efficacy of BXM against avian-origin A(H7N9) and A (H5N1) influenza viruses was evaluated.

Materials and methods

Compounds

BXM and BXA were synthesized at Shionogi & Co., Ltd. (Osaka, Japan). Oseltamivir acid (OSA) was purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). OSP was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Peramivir trihydrate (PRV) was purchased from AstaTech, Inc. (Philadelphia, PA, USA). FPV was purchased from PharmaBlock Sciences, Inc. (Nanjing, China).

Cells and viruses

The Madin-Darby canine kidney (MDCK) [European Collection of Cell Cultures (Public Health England, UK)] cells were maintained at 37 °C under 5% CO_2 in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin and 0.05% sodium hydrogen carbonate. Human lung adenocarcinoma epithelial (A549) cells were maintained at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin and 0.05% sodium hydrogen carbonate.

All tested viruses were propagated in embryonated chicken eggs and harvested from virus-containing allantoic fluids. Recombinant A/Anhui/1/2013 (H7N9) virus harboring NA/R292K and A/Hong Kong/483/1997 (H5N1) viruses [wild-type (WT), harboring NA/H275Y or asparagine-to-serine at amino acid position 294 (N294S)] were generated by plasmid-based reverse genetics (45). Recombinant viruses were propagated in embryonated chicken eggs and harvested from virus-containing allantoic fluids. Infectious titers were determined by the standard 50% tissue culture infectious dose ($TCID_{50}$) assay in MDCK cells.

Virus yield reduction assay

Two days prior to infection, MDCK cells were seeded in 96-well plates and the cells were infected with each virus at 100 $TCID_{50}$/well. The infected cells were incubated at 35 °C under 5% CO_2 for 1 hour and wash out the virus inoculum, followed by the addition of the fresh MEM including 2.5 μg/ml with or without [recombinant A(H5N1) viruses only] acetylated trypsin and defined concentrations of test compounds. BXA and FPV were dissolved in dimethyl sulfoxide and OSA was dissolved in distilled water. The cells were incubated at 35 °C under 5% CO_2 for 24 hours and viral titers in the culture supernatants were determined in MDCK cells. The 90% effective concentration (EC_{90})

values were finally calculated as the concentration necessary to decrease the viral titer in the culture supernatant to one-tenth of the untreated control value using a linear interpolation method. The mean and standard deviation (SD) were calculated from three independent experiments.

Genetic analysis

PA nucleotide sequences of 10,312 clinical isolates for A(H1N1), 13,185 for A(H3N2), 196 for A(H5N1) and 1,094 for A(H7N9), were downloaded from the National Center for Biotechnology Information (NCBI) and Global Initiative on Sharing All Influenza Data (GISAID) on October 24, 2018. Amino acid sequences of the PA protein were aligned using the ClustalW program in the component of Pipeline Pilot 2018 [BIOVIA. (San Diego, CA, USA)], and conservation of amino acid residues in close proximity to the BXA was calculated within individual influenza virus subtypes.

Animal experiments

Experiment 1

Six-week-old female BALB/c mice [Japan SLC, Inc. (Shizuoka, Japan)] were maintained in a temperature- and humidity-controlled environment. The mice were then intranasally infected (50 μL/mouse) with 4.0×10^5 $TCID_{50}$ [10.4 times of 50% mouse lethal dose (MLD_{50})] of A/Anhui/1/2013 (H7N9) strain or 75 $TCID_{50}$ (31.3 times of MLD_{50}) of A/Hong Kong/483/1997 (H5N1) strain under anesthesia (1.6 mg/mL zolazepam hydrochloride, 1.6 mg/mL tiletamine hydrochloride and 1.9 mg/mL xylazine hydrochloride in saline), and treatment started immediately, 24 [only A/Anhui/1/2013 (H7N9) infection] or 48 hours after virus inoculation. Subsequently, the mice were treated with BXM (0.5, 5 or 50 mg/kg/dose) twice daily (12 hours interval between each dosing) for 5 days through oral gavage. The dosing regimen of BXM 5 mg/kg/dose, twice daily for 5 days, is an extrapolated clinical setting from previous reports (46, 47). For the controls, the vehicle (0.5 w/v% methylcellulose) or OSP [5 (clinically-equivalent dose; 75 mg/kg/day in human or 50 mg/kg/dose)] was administered twice daily for 5 days through oral gavage (48). The dosing volume was 10 mL/kg and was calculated based on the body weight before each dosing. Survival rates and body weight changes were then monitored throughout a 21-day [A/Anhui/1/2013 (H7N9) infection] or 14-day [A/Hong Kong/483/1997 (H5N1) infection] period after the infection (n = 10/group). The viral titers in the lungs of mice at 1, 3 and 5 days post-infection (dpi) were finally determined in MDCK cells (n = 5/group), whereas viral titers in the brains and kidneys of mice [only A/Hong Kong/483/1997 (H5N1) infection] at 6 dpi were determined in MDCK cells

(n = 5/group).

Experiment 2

Under anesthesia, the mice were intranasally infected with 75 $TCID_{50}$ (31.3 MLD_{50}) of A/Hong Kong/483/1997 (H5N1) virus, and treatment started 48 hours after virus inoculation. The mice were treated with BXM (5 or 50 mg/kg/dose) twice daily for 5 days through oral gavage. For the controls, the oral gavage method was used to administer the vehicle or OSP (10 mg/kg/dose) twice daily for 5 days. Combination therapy was performed with BXM (5 mg/kg/dose) and OSP (10 mg/kg/dose) twice daily for 5 days through oral gavage. The dosing volume was 10 mL/kg and was calculated based on the body weight before each dosing. Subsequently, survival rates and body weight changes were monitored throughout a 21-day period after the infection (n = 5/group). The viral titers in the lungs, brains and kidneys of mice at 3, 5, 6 and 7 dpi were then determined in MDCK cells (n = 5/group).

Sequence analysis of the PA region in experiment 1

The viral RNAs derived from lung homogenates of BXM-treated mice were extracted by PureLink Viral RNA/DNA Mini Kit [Thermo Fisher Scientific, Inc. (Waltham, MA, USA)] according to the manufacturer's protocol. Reverse transcription reaction, amplification of complementary DNA and sequencing reaction were performed as previously reported (49). The primers used in this study were as follows; PA-1F, 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′ and PA-955R, 5′-TGCATTTGATTGCATCATATAG-3′. Sequence analysis of PA N-terminal domain [the PA gene of A/Anhui/1/2013 (H7N9) strain] was performed by the Sanger sequencing method using the 3500/3500xL genetic analyzer [Thermo Fisher Scientific, Inc. (Waltham, MA, USA)].

The viral RNAs derived from lung homogenates of BXM-treated mice were extracted by QIAamp® Viral RNA Mini Kit [QIAGEN (Hilden, Germany)] according to the manufacturer's protocol. Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) was used to quantify viral RNA in the obtained samples. Subsequently, Sanger sequencing was used to conduct sequence analysis of the PA region [the PA gene of A/Hong Kong/483/1997 (H5N1) strain]. All RNA samples quantified over the lower limit of quantification (800 copies/reaction) were subjected to this analysis. LSI Medience Corporation (Tokyo, Japan) conducted the RT-qPCR and Sanger sequencing.

Quantitative analysis of proinflammatory cytokines and chemokines

The collected lung samples in experimental 1 were homogenized, after which the levels of interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 in the lungs were quantitatively determined using Quantikine ELISA [R&D Systems (Minneapolis, MN, USA)] according to the manufacturer's protocol.

Histopathological experiments

Lungs were collected from mice infected with 75 $TCID_{50}$ (31.3 MLD_{50}) of A/Hong Kong/483/1997 (H5N1) strain at 5 dpi (n = 3/group). The samples were then fixed through perfusion in 10% phosphate-buffered formalin. The formalin-fixed left lungs were subsequently dissected, embedded in paraffin, and sectioned. Finally, hematoxylin and eosin (HE)-stained sections were prepared and subsequently used for histopathological analysis by Sapporo General Pathology Laboratory Co., Ltd. (Sapporo, Japan).

Combination effects of BXA and NAIs *in vitro*

The A549 cells, 319 $TCID_{50}$/well of A/Hong Kong/483/1997 (H5N1) virus, and compounds in serial dilutions (for BXA, 0.1-8.0 nmol/L; for OSA, 1.6-400 nmol/L; for PRV, 1.6-100 nmol/L) were simultaneously seeded in 96-well plates. The infected cells were then incubated at 37 °C under 5% CO_2 for 4 days. All supplements were prepared using MEM supplemented with 2% heat-inactivated FBS, 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin and 0.05% sodium hydrogen carbonate. After incubation, cell viability was assessed with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent, as previously reported (50). Subsequently, data analysis for yielding isobologram plots and calculating the combination index (CI) values was conducted as previously reported (42). In detail, CI values, under the condition that both substances were added at the concentration ratio of each 50% effective concentration (EC_{50}) value, were calculated using the following formula: $CI = (D_{A/A+B})/D_A + (D_{B/A+B})/D_B + (D_{A/A+B} \times D_{B/A+B})/(D_A \times D_B)$. The combination effect was determined according to the following criteria:
$CI \leq 0.8$, synergy; $0.8 < CI < 1.2$, additive; $1.2 \leq CI$, antagonism.

Statistical analysis

To compare the survival periods after infection between each BXM-treated group and vehicle- or OSP-treated groups in experiment 1 and 2, the log-rank test was performed. To compare the survival periods after infection between BXM/OSP

combination group and vehicle- or OSP-treated groups in experiment 2, the log-rank test was also performed. Dunnett's multiple-comparison test was performed for comparisons between viral titers in mouse tissues, cytokine and chemokine levels in lung tissue and lung wet weight-to-body weight ratios between each BXM-treated group, or between the BXM/OSP combination group and vehicle-treated or OSP-treated groups at each timepoint. All statistical analyses were conducted using the statistical analysis software SAS version 9.2 for Windows [SAS Institute. (Cary, NC, USA)]. *P* values < 0.05, < 0.01 and < 0.001 were considered statistically significant.

Ethics statement

The animal experiments were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval numbers 15-0063, 15-0067, 15-0068, 16-0107, 16-0108 and 18-0107), and performed according to the guidelines of this committee. The facilities where the animal experiments were conducted are certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animals were housed in self-contained units [Tokiwa Kagaku Kikai Co., Ltd. (Tokyo, Japan)] at the ABSL3 facilities of the Faculty of Veterinary Medicine, Hokkaido University, Japan. The mice were euthanized when they lost greater than 30% of their body weight compared with their pre-infection weight in each animal experiment.

Results

Inhibitory effect of BXA on viral replication of A(H7N9) and A(H5N1) *in vitro*

To examine the antiviral activity of BXA against H7 or H5 HPAIVs, isolated from 1997 to 2021, *in vitro* drug susceptibility of BXA was evaluated by using MDCK cells. BXA showed comparable inhibition efficacy in tested natural isolated (mean EC_{90} ranged from 0.7 to 1.5 nmol/L) compared with that of A/Anhui/1/2013 (H7N9) and A/Hong Kong/483/1997 (H5N1) strains as previously reported (**Table 1**). BXA also exhibited comparable potency against recombinant A/Anhui/1/2013 (H7N9) harboring NA/R292K or recombinant A/Hong Kong/483/1997 (H5N1) harboring NA/H275Y or NA/N294S substitution to the WT, indicating no-cross resistance with NAIs was observed. In contrast, OSA required approximately 70-fold or higher concentrations to archieve the same levels of inhibitory efficacy against NAI-resistant viruses as that of the WT virus. However, FPV showed comparable antiviral activity against the tested viruses, but the concentration range of FPV used remained higher than that of BXA. Among the PA amino acid polymorphisms, avian-origin influenza A(H7N9) viruses typically harbor a polymorphic alanine-to-serine at amino acid position 37 (A37S) in the PA, which is involved in BXA binding in the endonuclease domain (**Table 1 and 2**). However, this PA polymorphism was not associated with BXA susceptibility.

The protective efficacy of BXM on lethal A(H7N9) and A(H5N1) infections *in vivo*

In order to evaluate the effects of BXM against A(H7N9) and A(H5N1) viruses in a lethal infection model, mice were infected with 10.4 MLD_{50} of A/Anhui/1/2013 (H7N9) at first. All vehicle-treated mice died within 7 dpi (**Fig. 1a**). Clinically-equivalent dosing of OSP (48), 5 mg/kg twice daily for 5 days, and a supratherapeutic dose, 50 mg/kg twice daily for 5 days, resulted in 30% and 50% survival, respectively. In this setting, survival rates of BXM at 0.5, 5, and 50 mg/kg twice daily for 5 days were all 100%. Next, mice were infected with 31.4 MLD_{50} of A/Hong Kong/483/97 (H5N1). All vehicle- or OSP at 5 mg/kg-treated mice died within 7 and 9 dpi, respectively (**Fig. 1b**). By contrast, OSP at 50 mg/kg resulted in 70% survival. In this setting, survival rates of BXM at 0.5, 5, and 50 mg/kg were all 100%. These results indicate that BXM improves survival in mice infected with clinical isolates, A/Anhui/1/2013 (H7N9) and A/Hong Kong/483/97 (H5N1) strains.

Effects of BXM on viral titers in mice infected with A(H7N9) and A(H5N1) viruses

In order to examine the inhibitory effects of BXM on the viral replication of

A/Anhui/1/2013 (H7N9) strain *in vivo*, viral titers in lung homogenates derived from infected mice were measured at 1, 3 and 5 dpi. BXM treatment at 5 and 50 mg/kg decreased viral titers in the lungs of mice by more than 3-logs compared with that of vehicle- or OSP-treated group, whereas viral titers of all OSP-treated groups were comparable with those of vehicle-treated group at 1 dpi (**Fig. 2a**). Viral titers were suppressed by more than 1 to 3-logs following BXM treatment compared with that of vehicle- or OSP-treated groups at 3 and 5 dpi. In addition, mutation analysis of the PA N-terminal domain (residues 1 to 209) (51) of A/Anhui/1/2013 (H7N9) strain was performed on lung homogenates of the infected mice treated with BXM. There were no amino acid changes in the analyzed regions including residues implicated in BXA resistance by affecting BXA binding to the active center of the endonuclease domain (17, 20), such as PA/I38.

Next, the inhibitory effect of BXM on viral replication of A/Hong Kong/483/97 (H5N1) strain *in vivo* was examined. Compared with vehicle treatment, OSP treatment decreased viral titers in lung homogenates derived from infected mice at 1 dpi (**Fig. 2b**). However, this effect diminished in effectiveness at 3 and 5 dpi. In contrast, compared with vehicle- and OSP-treated groups, BXM-treated groups significantly decreased viral titers in the lungs of mice at 1 dpi and sustained this effectiveness until 5 dpi. Genotypic alterations in the whole PA gene of A/Hong Kong/483/97 (H5N1) derived from lung homogenates of infected mice treated with BXM were analyzed. In these lung samples, 3 sample had nucleic acid changes, but 2 out of 3 samples had silent mutation. The other sample had a substitution of isoleucine-to-valine at amino acid position 127 (I127V) in the PA, which did not impact on BXA susceptibility. In A(H5N1) HPAIV-infected mice model, infectious viruses were detected in organs other than respiratory tissues (e.g., brain and kidney), which is proposed to be due to the spillover of high viral titers of the virus replicated in mice (52). Therefore, the inhibitory effects on viral titers in the brain and kidney homogenates at 6 dpi, when viruses could be detected, were analyzed. As a result, infectious viruses were detected in the brains of all vehicle-treated mice, three of five OSP at 5 mg/kg-treated mice and one of five OSP at 50 mg/kg-treated mice (**Fig. 3a**). In contrast, no viruses were detected in the brain of BXM at 5 and 50 mg/kg-treated mice. However, infectious viruses in the kidney were detected only in the vehicle-treated mice (**Fig. 3b**). These results suggest that BXM has profound inhibitory effects on viral replication in the lungs of mice infected with A(H7N9) and A(H5N1) viruses. BXM also inhibited viral replication in the extra respiratory organs of A(H5N1) virus-infected mice.

Prevention of inflammation in mice lungs following BXM treatment

It has been previously reported that production of proinflammatory cytokines and chemokines, such as IL-6 and MCP-1, is markedly elevated in A(H7N9) or A(H5N1) virus-infected patients. Furthermore, inflammation in the lungs associated with A(H7N9) or A(H5N1) virus infection resulted in a rapidly progressive pneumonia and development of ARDS in the majority of hospitalized patients (31, 33, 34). Therefore, the impact of BXM treatment upon proinflammatory cytokines and chemokines production in the lung of mice infected with A/Anhui/1/2013 (H7N9) or A/Hong Kong/483/97 (H5N1) strain was evaluated. Treatment of the A(H7N9) virus-infected mice with BXM at 5 and 50 mg/kg resulted in a significantly less pronounced production of both proinflammatory cytokines and chemokines when compared with vehicle-treated mice, consistent with the reduction of viral titers in the lungs of mice (**Fig. 4a**). In contrast, OSP treatment showed a limited inhibitory effect on IL-6 and MCP-1 production in the lungs of mice compared with vehicle-treated mice. In case of A(H5N1) virus infection, BXM-treated group exhibited significantly less-pronounced production of IL-6 and MCP-1 compared with vehicle- or OSP at 5 mg/kg-treated groups, which resulted from the reduction of viral titers in mouse lungs at 5 dpi (**Fig. 4b**). OSP-treated group also exhibited a significant inhibitory effect on IL-6 and MCP-1 production in mice lungs compared with vehicle-treated group.

Subsequent analyses of proinflammatory cytokine and chemokine production in mice lungs, including histopathological findings from lungs of A(H5N1) virus-infected mice treated with BXM were performed next. Acute pneumonia, including thickening and inflammatory cell infiltration of alveolar walls, inflammatory cell infiltration within the alveoli, bronchiolar and perivascular cell infiltration and edema, was observed in about quarter to half of the global area of all vehicle-treated mice lungs (**Fig. 5**). These findings were suppressed in mice treated with OSP at 5 mg/kg compared with those treated with the vehicle. In contrast, no abnormal findings in the lungs were observed in each lung after BXM at 5 mg/kg treatment. These results demonstrate that BXM has a profound inhibitory effect on inflammation in the lungs of A(H7N9) or A(H5N1) virus-infected mice.

Delayed treatment of BXM for lethal A(H7N9) virus infection

To further investigate the therapeutic effect of BXM, mice infected with 10.4 MLD_{50} of A/Anhui/1/2013 (H7N9) were treated with BXM starting at 24 and 48 hours after infection, and subsequently survival and body weight loss were monitored. Mice without treatment died from 5 to 7 days after infection, consistent with findings presented

in Fig. 1a (**Fig. 6**). In this setting, the 24 hours delayed treatment of BXM at 5 and 50 mg/kg resulted in complete protection against lethal infection with A/Anhui/1/2013 (H7N9) strain. The therapeutic effect was still observed with the mice given BXM at 48 hours after infection. A time-dependent protective effect by means of body weight change was confirmed on all BXM-treated mice. These results suggest that BXM exhibits therapeutic effects against A/Anhui/1/2013 (H7N9) in mice when BXM treatment is initiated no later than 48 hours after infection.

Combination efficacy of BXM and NAIs on A(H5N1) virus infections

The combination efficacy of BXM and OSP in A(H5N1) virus-infected mice was next evaluated to explore the possibilities of a potent therapeutic option compared with BXM monotherapy. First, combination effects of BXA and NAIs (OSA or PRV) on A(H5N1) virus infection *in vitro* were evaluated. The cytopathic effect (CPE) assay was then conducted in A549 cells. The isobologram plot showed that the combination of BXA with OSA and PRV resulted in CI values of 0.19 and 0.40, respectively, indicating that BXA exhibited synergistic effects with NAIs *in vitro* (**Fig. 7**), as previously reported (42, 53). Next, to investigate the therapeutic effect of BXM monotherapy (5 or 50 mg/kg twice daily for 5 days) or combination therapy at clinically higher doses of OSP (10 mg/kg twice daily for 5 days) (54), A/Hong Kong/483/1997 (H5N1)-infected mice were treated, starting at 48 hours after infection. All mice treated with vehicle and OSP at 10 mg/kg-treated mice showed gradual body weight loss after viral infection, resulting in death within 8 dpi (**Fig. 8**). Further, mice treated with BXM at 5 mg/kg showed delayed body weight loss. However, 40% of BXM at 5 mg/kg-treated mice survived at 21 dpi. Additionally, mice treated with BXM at 50 mg/kg showed little body weight loss and survived. In this setting, it is confirmed that BXM (5 mg/kg)/OSP (10 mg/kg) combination therapy suppressed body weight loss and improved survival at 21 dpi compared with monotherapy. Furthermore, all BXM monotherapy groups showed significantly lower viral titers in the lungs of mice compared with vehicle- and OSP-monotherapy groups (**Fig. 9a**). Notably, BXM/OSP combination therapies also reduced viral titers in the lungs and tended to reduce viral titers at 6 and 7 dpi more than those of BXM at 5 mg/kg monotherapy. Furthermore, BXM monotherapy or BXM/OSP combination therapy significantly reduced viral titers in the brains and kidneys at 5, 6 and 7 dpi compared with vehicle or OSP treatment (**Fig. 9b and 9c**).

Discussion

In this study, BXA exhibited similar *in vitro* activity against H7 or H5 variants and A(H7N9) and A(H5N1) viruses harboring NAI-resistant mutants in comparison with seasonal and other zoonotic strains (18, 19). BXA was susceptible to these viruses and showed no cross resistance to NAI-resistant mutants, adding to the evidence that it is effective against A(H7N9) and A(H5N1) viruses, which pose a risk for human infection (4-6). Several A(H7N9) viruses possesses PA/A37S polymorphism, which is involved in BXA binding in the endonuclease domain. However, PA/A37S did not impact on BXA susceptibility.

The therapeutic effect of oral BXM administration was investigated following A(H7N9) or A(H5N1) challenge in a lethal infection murine model. In previous reports, OSP had limited effect on the inhibition of body weight loss and the concomitant reduction of viral titers in mice infected with A/Anhui/1/2013 (H7N9) and A/Hong Kong/483/97 (H5N1) strains, comparable results as above were obtained in this study (28, 33). In this model, BXM achieved strong reductions in viral titers compared with the vehicle and OSP-treated groups and resulted in significant improvements in mortality. Especially, BXM at 5 and 50 mg/kg continued to suppress the viral load from the day after administration. The target plasma concentration 24 hours after a single-dose (C_{24}) of BXA be set at 6.85 ng/mL in non-clinical and clinical studies (46, 47). The concentration of BXA in plasma could be maintained above the target concentration of 6.85 ng/mL for at least 5 days following oral administration of BXM at 40 mg in humans (47). The target plasma concentration 120 hours after a single-dose (C_{120}) of BXA after 5-day dosing of BXM at 5 mg/kg twice daily in mice are expected to be close to those in humans (46, 47); therefore, the dosages used in mice model of BXM at 5 mg/kg twice daily for 5 days are comparable to the clinical dosages. BXM also extended the treatment window, which indicated that BXM might be a reasonable option in an infection with A(H7N9) and A(H5N1) viruses. These results suggest that BXM treatment, using the extrapolated clinical setting from the above mice models, has therapeutic potential against A(H7N9) or A(H5N1) virus infection in mammals.

In human infection with A(H7N9) and A(H5N1) viruses, virus-induced proinflammatory cytokine and chemokine dysregulation in the lungs or serum contributes to disease severity (55, 56). Similar findings were observed in A(H7N9) and A(H5N1) virus-infected mice. Proinflammatory cytokines and chemokines are highly induced in the early phase of influenza virus infection and are associated with airway inflammation; thus, BXM may ameliorate severe influenza pneumonia due to exerting an inhibitory

effect upon the production of IL-6 and MCP-1 in the early stages of virus infection (32, 57). Early inhibition of viral replication is more promising than inhibition of the cytokine response for improving host survival during A(H5N1) virus infection (58). Moreover, the polymerase complex genes of human A(H5N1) strains contribute to high pathogenesis in mammals (59). Some novel polymerase inhibitors (e.g., FPV and pimodivir) have also shown strong efficacy for inhibiting viral replication and preventing death in A(H5N1) virus-infected mice compared with OSP, implying that the inhibition of polymerase activity was effective in treating A(H5N1) infection (60, 61). Therefore, BXM, which is more potent for inhibiting viral RNA transcription and thereby inhibiting viral replication, is proposed to be more suitable as a therapeutic option than OSP.

Next the effects of BXM in A(H5N1) HPAIV-infected murine model were evaluated in detail. A(H5N1) HPAIVs have an ability to replicate and spread in primary human immune cell cultures or multiple organs of mice or ferrets, unlike seasonal strains (62, 63). Therefore, it is meaningful to clarify the inhibitory efficacy of BXM on the reduction of viral load in multiple organs and assess mortality caused by A(H5N1) HPAIV infection in mice models. In this A/Hong Kong/483/97 (H5N1)-infected mice model, BXM at 5 mg/kg was sufficient to significantly decrease the viral load in the lungs, resulting in significant improvements in mortality compared with vehicle or OSP. Viral replication in the brains or kidneys was detected by spillover because of high viral burden in mice (64, 65). The systemic spread of A(H5N1) has also been reported to be associated with disease pathogenesis in mammals, particularly for A/Hong Kong/483/1997 (H5N1) strain, which accounts for neurotropism (51, 63, 66, 67). Similarly, few viruses were detected in the brains and kidneys after BXM treatment, suggesting that BXA directly inhibited viral replication in tissues other than the respiratory organs. Additionally, the antiviral efficacy of BXM has first been observed in the lungs, which is proposed to contribute to the inhibition of viral spread from the lungs to extra respiratory organs. This finding evidences that BXM treatment, using the extrapolated clinical setting from the above mice models, has therapeutic potential against H5 HPAIVs, with high mortality being observed in mammals.

BXM/OSP combination therapy is a beneficial option for reducing viral titers in the lungs and improving mortality in severe A(H1N1)-infected mice model (42). In this study, combination therapy increased the survival of mice compared with BXM- or OSP-monotherapy in A(H5N1)-infected mice model, suggesting that combination therapy was effective without antagonism for treating A(H5N1) infection. BXM and OSP have different modes of action on the viral replication cycle. Therefore, it is considered that the combination therapy showed a strong viral inhibition effect and reduced systemic

viral loads. Combination therapy with the polymerase inhibitor FPV in addition to OSP started at 96 hours after infection resulted in the complete suppression of mortality in A(H5N1)-infected mice (44), however in this study, BXM/OSP combination started at 48 hours after infection did not completely suppress mortality. This was proposed to be due to the higher infectivity of titers infected in this study compared with those used in previous studies (43, 44). Nevertheless, these results present the first evidence confirming the efficacy of combination therapy with BXM and OSP for treating A(H5N1) HPAIV infection.

Summary

Avian-origin A(H7N9) and A(H5N1) viruses sporadically infect humans from birds with high morbidity and mortality by highly severe respiratory dysfunction. Although NAIs are recommended for the treatment of these viral infections, case reports are not enough, and the emergence of NAI-resistant viruses is a concern. BXM, which is converted metabolically to its active form BXA, is an orally available CEN inhibitor approved for clinical use in adults and adolescents worldwide. BXA selectively inhibits CEN activity carried by the PA protein, which is highly conserved regardless of influenza virus subtypes, of influenza A and B viruses and shows broad antiviral activities against several influenza viruses. However, there were few reports in which BXA were treated for animals with avian influenza virus infection; therefore, it was considered that obtaining the evidence was crucial.

In this study, BXA susceptibility of A(H7N9) and A(H5N1) viruses isolated over several years naturally and those recombinant viruses harboring NAI-resistant mutations was retained. As observed, the oral administration of BXM drastically decreased viral loads in the lungs and extra respiratory organs. BXM administration also reduced lung inflammation and improved mortality in an A(H7N9) and A(H5N1)-infected mice model. Moreover, combination therapy with BXM and OSP can be used as a treatment option for severe influenza such as A(H7N9) and A(H5N1) infections. Furthermore, compared with monotherapy, combination therapy with BXM and OSP had more potent effects on viral replication in organs, thereby improving survival in 48 hours delayed treatment model. These findings indicate that BXM has potent antiviral activity against H7 and H5 influenza virus infection.

Chapter II

Characterization of PA amino acid polymorphisms on the susceptibility of zoonotic influenza A viruses to baloxavir acid

Introduction

Genetic reassortment of IAVs through cross-species transmission can contribute to the generation of pandemic influenza viruses (68). IAVs kept in aquatic wild birds infect humans *via* epidemics of poultry and livestock when they efficiently adapt and replicate because the segmented RNA genome of IAVs allows genetic reassortment frequently; thereby, new viruses can emerge (3).

BXA targets CEN located in the PA N-terminal domain, which is highly conserved among IAVs (**Table 2**), and it exhibits viral replication inhibitory activity against various IAVs, including zoonotic strains such as H5 and H7 subtypes, at lower concentrations than other anti-influenza drugs (18, 19). However, variants exhibiting reduced BXA susceptibility have been detected in some seasonal influenza patients who received BXM therapy (e.g., PA/I38T, PA/I39F or PA/I38M) (17, 20-22). Several studies have examined the impact of PA/I38 substitution on the fitness of various seasonal influenza virus strains (18, 22, 69, 70). However, many characteristics of zoonotic influenza viruses remain unclear because natural polymorphisms at this residue are rare. In a previous study, only two A(H5N1) virus isolates harboring PA/I38T [A/chicken/Michigan/22-013961-001/2022 (chicken/MI/22)] and PA/I38M [A/Cooper's hawk/Minnesota/22-012931-001/2022 (hawk/MN-1/22)] were tested and showed lower BXA susceptibility compared with baseline susceptibility, where A(H5N1) viruses lacking the flagged PA substitutions were used to determine the median baseline EC_{50} (24). However, the evidence cannot prove the responsible amino acid substitutions for reduced BXA susceptibility, because tested isolates have several amino acid substitutions except for PA/I38 [for example, chicken/MI/22 have I38T, methionine-to-isoleucine at amino acid position 61 (M61I), and alanine-to-threonine at amino acid position 85 (A85T) in the PA domain]. This means that the impact of other polymorphisms differing from PA/I38T on the BXA susceptibility is unknown. Moreover, unlike the case of NAIs (e.g., NA/H275Y or NA/R292K at N2 numbering), genetic markers of BXA susceptibility in zoonotic influenza viruses have been unclear.

In this chapter, the BXA susceptibility of recombinant A(H5N1) viruses harboring single PA/I38F, M, or T substitutions, in addition to various avian and swine strains with PA polymorphisms was evaluated. Moreover, to assess the impact of PA/I38 substitutions on BXA susceptibility and replicative fitness, recombinant A/Hong Kong/483/97 (H5N1) strains harboring individual substitutions were generated.

Materials and methods

Compounds

BXA was synthesized at Shionogi & Co., Ltd. (Osaka, Japan). OSA was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). FPV was supplied from PharmaBlock Sciences, Inc. (Nanjing, China).

Cells and viruses

MDCK cells were maintained at 37 °C under 5% CO_2 in MEM supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 0.05% sodium hydrogen carbonate. Recombinant A/Hong Kong/483/1997 (H5N1) viruses (WT and harboring single PA/I38F, M and T substitutions) were generated and propagated by plasmid-based reverse genetics (45). The avian and swine IAVs tested in this study (total of 28 strains) were selected considering isolation areas, subtypes, isolation dates and PA amino acid polymorphisms (**Table 3, 4 and 6-8**). These viruses were propagated in embryonated chicken eggs and harvested from virus-containing allantoic fluids. Infectivity titers were determined by $TCID_{50}$ assays in MDCK cells. The amino acid sequences in the PA N-terminal region of recombinant A/Hong Kong/483/1997 (H5N1) viruses and other avian or swine IAVs tested in this study were determined by the Sanger sequence method and PA polymorphisms were detected in some strains (**Table 3 and 4**).

Virus yield reduction assay

As described in the Materials and methods part of chapter I.

Genetic analysis

PA nucleotide sequences for human, avian, and swine influenza viruses collected between January 1, 2012, and September 21, 2022 (total: 41,537), were obtained from NCBI and GISAID on September 21, 2022, and aligned using GENETYX® ver. 14.0 for Windows [GENETYX Corp (Tokyo, Japan)].

Evaluation of virus replicative fitness

MDCK cells (30,000 cells/well) were seeded in 96-well plates 1 day prior to infection. Cells were infected with 100 $TCID_{50}$/well of each recombinant virus. The infected cells were incubated at 35 °C under 5% CO_2 for 1 hour and then washed with MEM, followed by the addition of fresh MEM and further incubation at 35 °C under 5%

CO_2. Cell culture supernatants were collected at indicated time points, and viral titers (log_{10} $TCID_{50}$/mL) were determined by $TCID_{50}$ assay in MDCK cells. Viral titers were calculated based on a virus-induced CPE and expressed as log_{10} $TCID_{50}$/mL.

Statistical analysis

Differences in titer between the WT virus and mutants harboring the PA/I38F, M or T substitutions at each time-point were examined using Welch's *t*-test. Statistical analyses were conducted using the statistical analysis software SAS, version 9.4 for Windows. *P* values < 0.05, < 0.01 and < 0.001 were considered statistically significant.

Ethics statement

All experiments were authorized by the Biosafety Management Committee on Pathogens and Other Hazardous Agents and the Safety Committee on Genetic Recombination Experiments, Hokkaido University. Regarding dual use experiments, the experiment plan, the progress of the experiment and its safety assurance are described, and the contents have been approved by this committee.

Results

BXA susceptibility and replicative fitness of recombinant A/Hong Kong/483/97 (H5N1) with PA variants

In order to assess the impact of PA/I38 substitutions on BXA susceptibility and replicative fitness, the recombinant A/Hong Kong/483/97 (H5N1) strains harboring the individual substitutions were generated and subjected to testing of susceptibility and replicative fitness in MDCK cells. Compared with the recombinant WT virus, which had a mean EC_{90} value of 1.1 nmol/L, PA/I38F, M and T variants increased EC_{90} values by 24.0-, 15.5- and 48.2-fold, respectively (**Table 5**). In contrast, OSA and FPV showed comparable inhibitory activity against each virus. Replicative capacity of each PA-substituted virus was significantly lower than that of WT virus in MDCK cells at 24 hours post-infection and each virus replicated to lower titers at all time points compared with WT virus (**Fig. 10**). These data indicate that PA mutants, especially PA/I38T strain, had significantly decreased BXA susceptibility and impaired fitness compared with WT virus. PA mutants also showed no cross-resistance to OSA and FPV, which have different mechanisms of action from BXA, combined with the results of previous susceptibility tests against recombinant A/Hong Kong/483/97 (H5N1) harboring NA/H275Y (**Table 1**).

BXA susceptibility of temporally and geographically distinct avian and swine influenza viruses

As previously reported, H1, H5, H7 and H9 subtype zoonotic influenza viruses were susceptible to BXA *in vitro*, as well as to H1 and H3 subtype human clinical isolates (18, 19). For robust characterization of the broad-spectrum of BXA activity, drug susceptibility tests were performed against avian and swine IAVs. The median EC_{90} values of BXA were 1.6 nmol/L for avian and swine strains, respectively (**Table 6 and 7**). Among the PA amino acid polymorphisms, PA/I38 variants were rare, while alanine-to-threonine at amino acid position 20 (A20T), tyrosine-to-histidine at amino acid position 24 (Y24H), and A37S were harbored in more than 1% of all viruses analyzed in the database (**Table 8**). The amino acid polymorphisms, A20T, Y24H, and A37S, implicated in BXA binding to the PA endonuclease domain, did not impact on BXA susceptibility (**Table 6 and 7**). The median EC_{90} values of FPV were 30433.5 nmol/L and 13957.1 nmol/L for avian and swine strains, respectively. These data indicate that all tested viruses, regardless of isolation areas, subtypes, isolation date and PA amino acid polymorphisms were susceptible to BXA, which is comparable to that of IAVs, as previously reported.

Discussion

PA/I38F, M, and T viruses isolated from BXM-treated patients and natural variants such as PA/I38M, PA/I38L, and glutamic acid-to-glycine at amino acid position 23 (E23G) exhibit reduced BXA susceptibility in humans (22, 23, 71). These polymorphisms have been implicated as playing a role in the binding of BXA to the PA endonuclease domain (17). However, database analyses indicated a rare occurrence of PA/I38 substitutions in animals; therefore, the BXA susceptibility of these isolates has not been determined. Recently, the susceptibility of A(H5N1) viruses harboring PA/I38T, I38M and A37T to BXA was reported (24), where BXA susceptibility was similar to that of seasonal influenza viruses. However, the impact on single PA/I38 substitution of other zoonotic influenza viruses and the replicative fitness of these were still unknown. Therefore, the impact of three major PA/I38 substitutions on reduced BXA susceptibility, I38T, F and M, was examined. These mutations resulted in lower BXA susceptibility, and the degree of reduction in BXA susceptibility was comparable to that of seasonal viruses (17, 72, 73). The structural changes of the active site of PA endonuclease domain by PA/I38T substitution in seasonal A(H1N1)pdm09 has weaken BXA binding to that, resulting in reduced BXA susceptibility (17). Notably, A(H5N1) and seasonal A(H1N1)pdm09 and A(H3N2) viruses exhibited similar X-ray crystal structures of the CEN active site and its amino acid sequence (51, 74). These results support decreased BXA susceptibility in PA/I38 substituted seasonal IAVs and A/Hong Kong/483/97 (H5N1) strains and PA/I38 substitutions could be potential genetic markers for BXA susceptibility of zoonotic strains.

Seasonal IAVs with PA/I38 mutations, especially PA/I38F or T, exhibit reduced fitness in MDCK cells (17, 72, 73), whereas variants with PA/I38M or T substitutions exhibit fitness comparable to that of the WT virus (71, 75). In this experiment, compared with the WT virus, the PA/I38T mutant had the most significantly impaired fitness, whereas the PA/I38F and PA/I38M mutants exhibited a trend of impairment in fitness in zoonotic IAVs. These observations suggest that H5 viruses harboring PA/I38 substitutions are less fit than seasonal strains. The PA/I38 substitution caused impaired CEN activity in IAVs (17), suggesting that the CEN activity of A(H5N1) with PA/I38 substitutions was impaired, resulting in decreased viral growth.

Previous reports indicated that several IAVs isolated from animals were susceptible to BXA (18, 19), but to date, there are no reports of PA polymorphisms associated with decreased BXA susceptibility. Therefore, the BXA susceptibility of avian and swine strains harboring various PA amino acid polymorphisms isolated in several

years, areas and subtypes was evaluated. All tested strains were susceptible to BXA, suggesting that no amino acid polymorphisms associated with reduced BXA susceptibility were identified. Genetic analysis of the amino acid residues involved in BXA binding to the PA endonuclease domain conducted for a decade revealed that amino acid polymorphisms PA/A20T, Y24H, and A37S were present in >1% of the isolates. These PA polymorphisms were not associated with BXA susceptibility; however, more studies are needed to evaluate other existing polymorphisms.

Summary

Genetic reassortment of IAVs in birds, pigs, and humans poses the potential risk of pandemics. Surveillance of animal-derived IAVs is important for influenza pandemic preparedness; however, the susceptibility of zoonotic IAVs to BXA is poorly characterized. In clinical settings, PA/I38 substitutions in seasonal IAVs reduce BXA susceptibility; however, PA amino acid polymorphisms at position 38 are rarely seen in zoonotic strains. Therefore, the characterization of PA/I38 substitutions in zoonotic strains has not been sufficiently analyzed, and there are little reports regarding amino acid polymorphisms in PA protein except PA/I38 that can serve as markers for BXA resistance mutations.

The recombinant A(H5N1) viruses harboring single PA substitutions, I38T, F and M, were less susceptible to BXA than WT, but were susceptible to OSA and FPV. PA mutants also exhibited significantly impaired replicative fitness in MDCK cells at 24 hours post-infection than that of WT virus. Moreover, to investigate new genetic markers for BXA susceptibility, geographically and temporally distinct IAVs isolated worldwide from birds and pigs were screened; however, BXA exhibited antiviral activity against avian and swine viruses at levels similar to those of seasonal isolates. A certain number of PA amino acid polymorphisms, A20T, Y24H and A37S, were detected in IAVs but did not affect BXA susceptibility. These data suggest that BXA exhibits broad-spectrum antiviral activity against IAVs in birds and pigs circulating worldwide. Although PA/I38 is highly conserved among recently isolated IAVs, continuous monitoring of PA amino acid polymorphisms in animal-derived influenza viruses is required.

Table 1. Antiviral activities of BXA and reference compounds against H7 or H5 influenza viruses isolated from humans and birds in a yield reduction assay using MDCK cells.

[a]Highly pathogenic avian influenza virus. Data represent the mean and SD from three independent experiments in MDCK cells.

	EC_{90} (nmol/L)					
	BXA		OSA		FPV	
Strain name	Mean	SD	Mean	SD	Mean	SD
A/Anhui/1/2013 (H7N9)	0.8	0.4	12.9	4.8	18,454.5	14,157.3
A/duck/Japan/AQ-HE28-3/2016 (H7N9)	0.6	0.1	38.5	18.2	12,014.4	7,990.3
A/duck/Japan/AQ-HE29-22/2017 (H7N9)[a]	0.7	0.3	12.9	9.9	17,192.0	13,933.2
A/duck/Japan/AQ-HE30-1/2018 (H7N3)[a]	1.1	0.6	27.9	18.0	18,879.8	13,666.6
A/ruddy turnstone/Delaware/103/2007 (H5N1)	1.4	1.3	12.8	5.7	16,927.4	12,375.0
A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)[a]	1.5	0.3	20.7	10.9	11,689.6	7,333.8
A/whooper swan/Mongolia/2/2006 (H5N1)[a]	0.9	0.5	14.9	1.6	13,812.0	11,056.8
A/black swan/Akita/1/2016 (H5N6)[a]	0.8	0.5	20	9.7	50,156.1	69,943.5
A/northern pintail/Hokkaido/M13/2020 (H5N8)[a]	1.3	1.0	19.5	11.9	16,025.5	10,164.5
A/whooper swan/Fukushima/0701B002/2021 (H5N8)[a]	1.1	1.0	19.2	13.9	16,052.1	12,507.3
A/whooper swan/Miyagi/0402B001/2021 (H5N8)[a]	0.7	0.3	11.3	2.8	6,992.6	1,370.8

Table 1. (continued)

[b]Virus generated by reverse genetics.

	EC_{90} (nmol/L)					
	BXA		OSA		FPV	
Strain name	Mean	SD	Mean	SD	Mean	SD
A/Anhui/1/2013 NA/R292K (H7N9)[b]	1.1	0.5	142,389.8	6,601.0	17,025.8	4,221.2
A/Hong Kong/483/1997 (H5N1)[b]	1.6	1.0	16.4	11.4	26,948.7	5,081.5
A/Hong Kong/483/1997 NA/H275Y (H5N1)[b]	3.2	1.2	4,054.9	1,295.7	31,129.5	11,788.5
A/Hong Kong/483/1997 NA/N294S (H5N1)[b]	1.7	0.4	1,291.2	482.6	77,002.6	2,190.5

Table 2. Amino acid polymorphisms of the BXA binding domain in PA from human and avian IAVs.

[a]The indicated amino acids have been previously shown to be involved in BXA binding to the active center of the endonuclease domain in the PA subunit (residues 20, 24, 34, 37, 38, 41, 80, 108, 119, 130, and 134) and associated with reduced susceptibility to BXA (residues 23, 37, 38 and 199), as reported previously (17). Amino acids different from the consensus sequence of human IAVs are highlighted in boldface and underlined. [b]The consensus sequences were determined by alignment analysis with the full-length PA nucleotide sequences obtained from NCBI and GISAID on October 24, 2018. Each number shown in parentheses represents the frequency (%) of the most frequent variants among 1,094, 10,312, 13,185 and 196 of PA nucleotide sequences from H7N9, H1N1, H3N2 and H5N1, respectively.

		PA amino acid position[a]												
Strain name	Subtype	20	23	24	34	37	38	41	80	108	119	130	134	199
A/Anhui/1/2013	H7N9	A	E	Y	K	**S**	I	H	E	D	E	Y	K	E
A/duck/Japan/AQ-HE28-3/2016	H7N9	A	E	Y	K	**S**	I	H	E	D	E	Y	K	E
A/duck/Japan/AQ-HE29-22/2017	H7N9	**T**	E	Y	K	**S**	I	H	E	D	E	Y	K	E
A/duck/Japan/AQ-HE30-1/2018	H7N3	A	E	Y	K	A	I	H	E	D	E	Y	K	E
Human influenza viruses isolated (As of October 24, 2018)[b]	H7N9	A (91.5)	E (100)	Y (100)	K (100)	**S** (99.9)	I (100)	H (100)	E (100)	D (100)	E (100)	Y (100)	K (100)	E (99.8)
	H1N1	A (99.3)	E (100)	Y (99.9)	K (100)	A (100)	I (99.9)	H (100)	E (100)	D (100)	E (100)	Y (100)	K (100)	E (100)
	H3N2	A (98.3)	E (100)	Y (100)	K (100)	A (100)	I (100)	H (100)	E (100)	D (100)	E (100)	Y (100)	K (100)	E (100)
	H5N1	A (85.2)	E (100)	Y (100)	K (100)	A (100)	I (100)	H (100)	E (100)	D (100)	E (100)	Y (100)	K (100)	E (100)

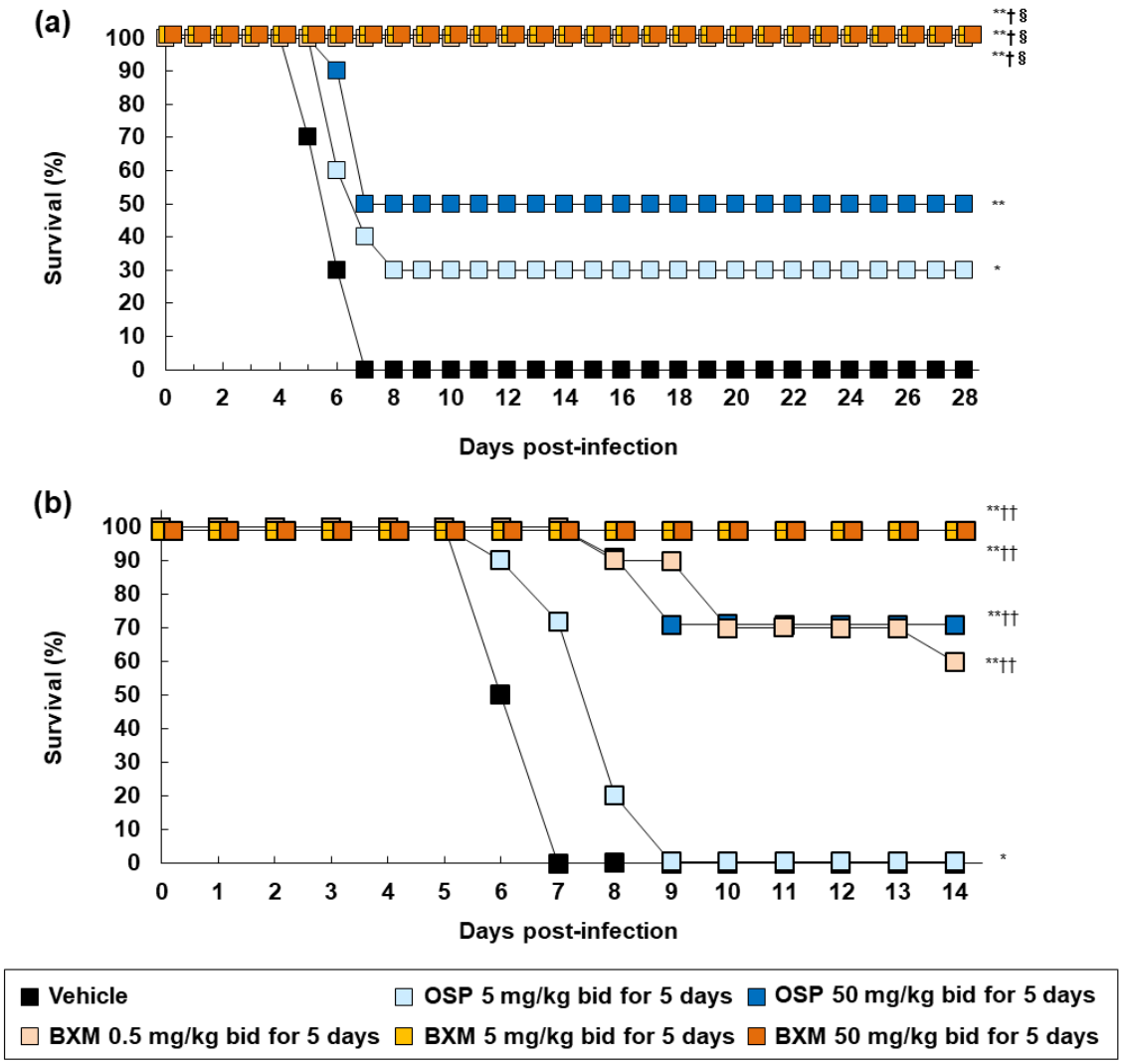

Figure 1. Therapeutic effects of BXM on survival in a lethal infection model of mice infected with A(H7N9) or A(H5N1) viruses.

Mice were intranasally infected with (a) 4.0×10^5 $TCID_{50}$ (10.4 MLD_{50})/mouse of A/Anhui/1/2013 (H7N9) or (b) 75 $TCID_{50}$ (31.4 MLD_{50})/mouse of A/Hong Kong/483/97 (H5N1), and treatment was started immediately after virus inoculation (n = 10/group). Survival time was monitored through a (a) 28-day or (b) 14-day period after the infection. Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily. The log-rank test was applied for comparison of the survival time between each group (*$p < 0.05$, **$p < 0.001$ compared with vehicle, †$p < 0.01$, ††$p < 0.001$ compared with OSP at 5 mg/kg, §$p < 0.05$ compared with OSP at 50 mg/kg).

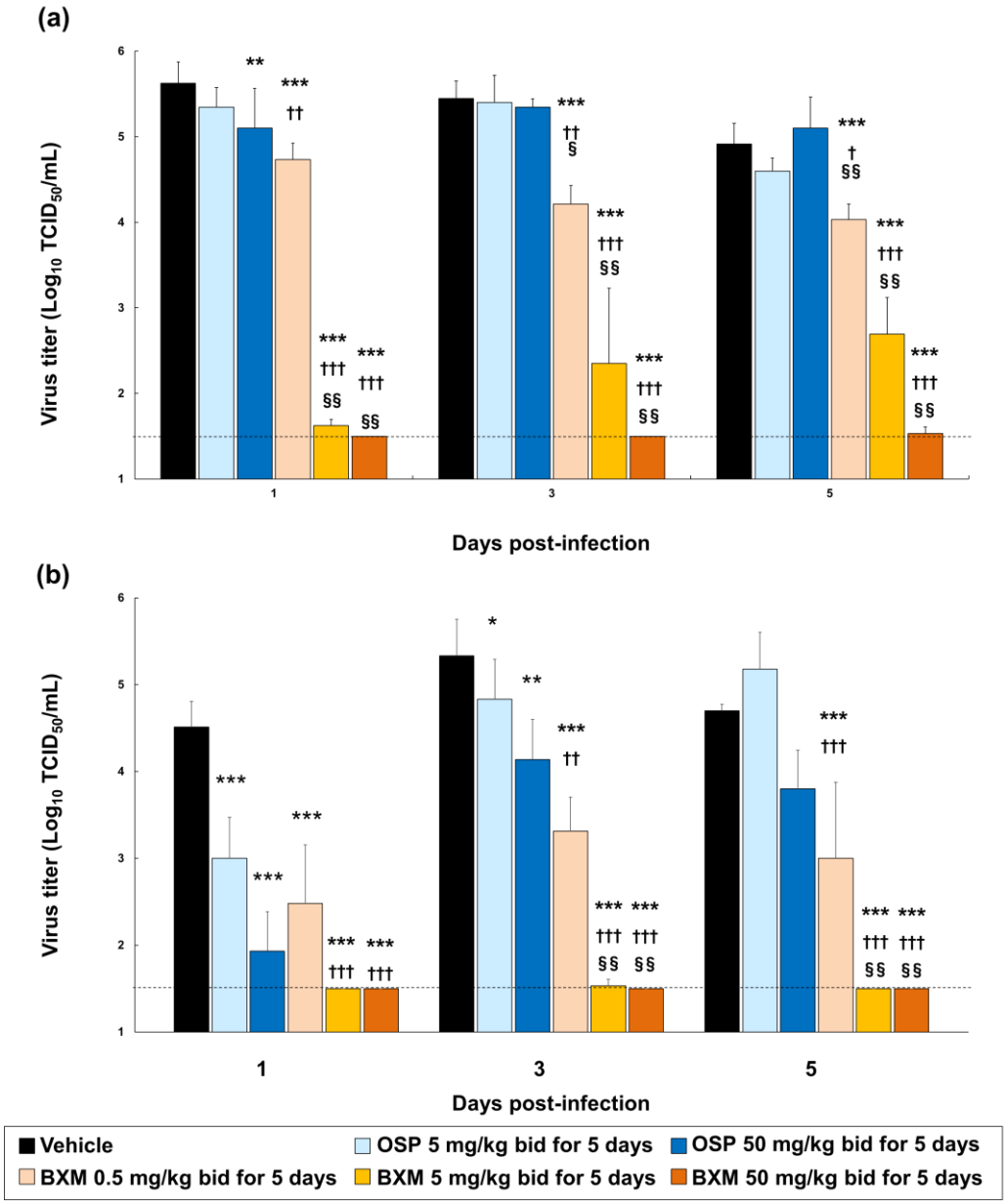

Figure 2. Inhibitory effect of BXM on viral titers in the lungs of mice infected with A(H7N9) or A(H5N1) virus.

Mice were intranasally infected with (a) A/Anhui/1/2013 (H7N9) or (b) A/Hong Kong/483/97 (H5N1) and treatment was started immediately after virus inoculation (n = 5/group). The viral titers ($TCID_{50}$) in the lungs of mice at 1, 3 and 5 dpi were measured in MDCK cells. The bars represent the mean viral titers + SD. The lower limit of quantification of the viral titer is indicated by a dotted line (1.5 Log_{10} $TCID_{50}$/mL). Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily. Dunnett's multiple-comparison method was conducted for statistical comparison (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle, †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$ compared to OSP at 5 mg/kg, §$p < 0.01$, §§$p < 0.001$ compared to OSP at 50 mg/kg).

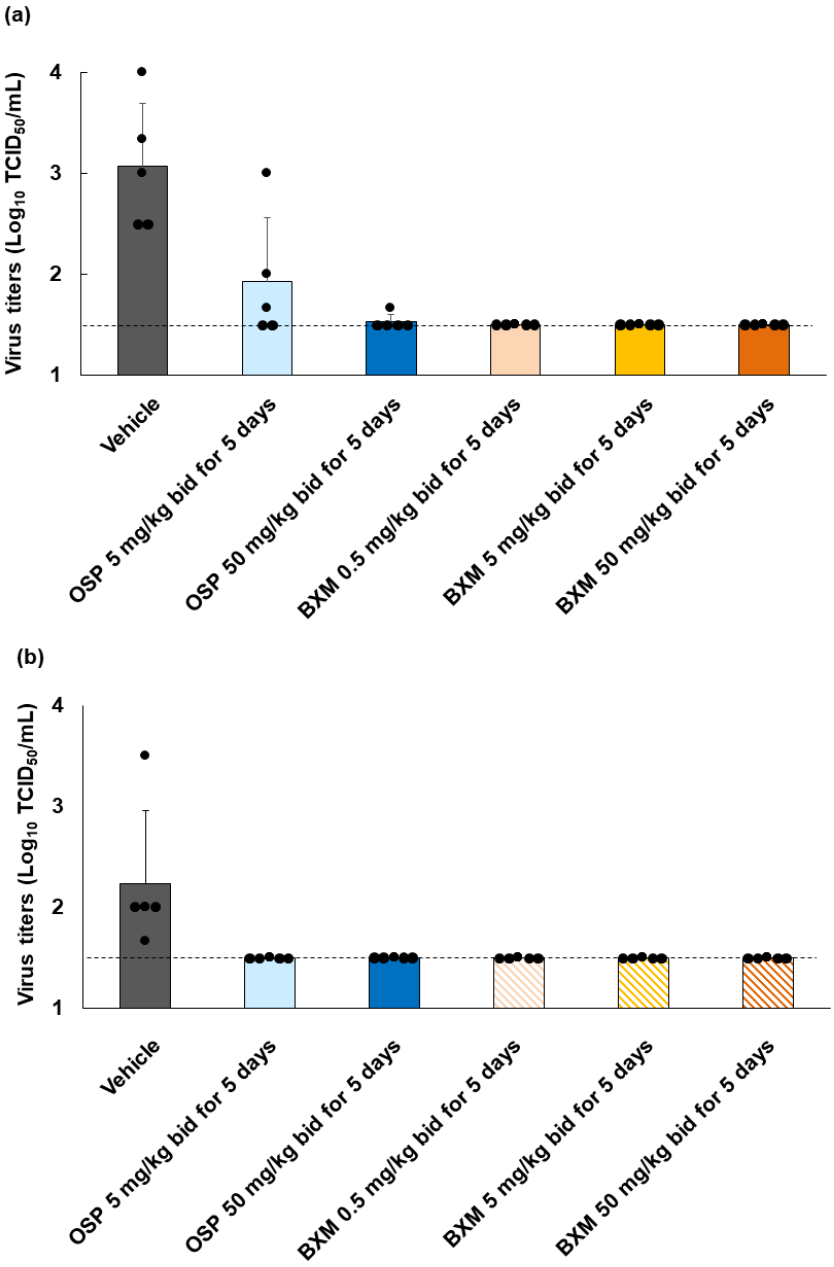

Figure 3. Inhibitory effects of BXM on viral titers in the brains or kidneys of mice infected with A(H5N1) virus.

The mice were intranasally infected with A/Hong Kong/483/1997 (H5N1), and then treatment was started immediately after virus inoculation (n = 5/group). The viral titers in mice (a) brains or (b) kidneys at 6 dpi measured in MDCK cells. The bars represent the mean viral titers + SD per group and individual viral titers for each sample are shown as black circles. The lower limit of quantification of the viral titer is indicated using a dotted line (1.5 $\log_{10}$ $TCID_{50}$/mL).
Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily.

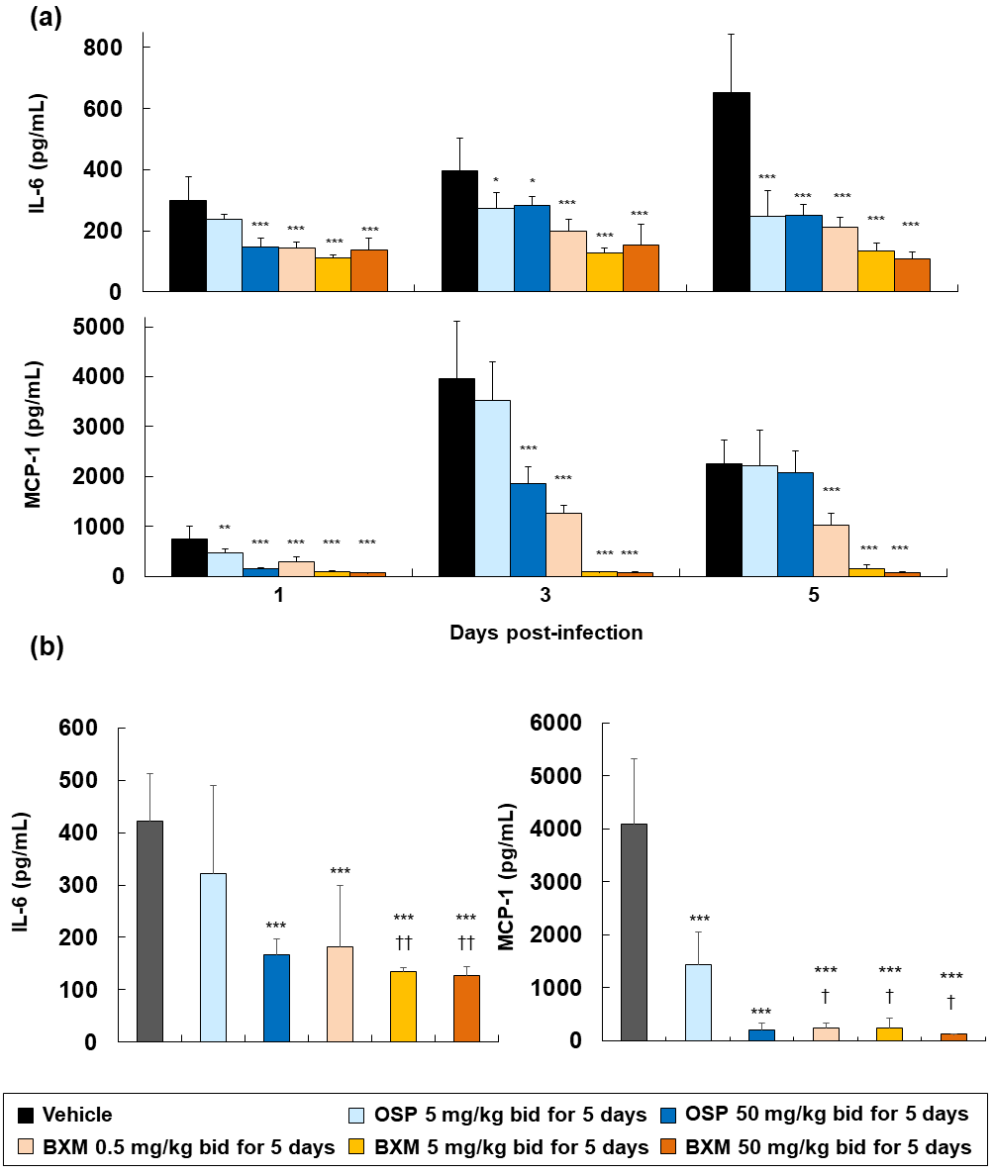

Figure 4. Suppressive effects of BXM on proinflammatory cytokine and chemokine production in the lungs of mice infected with A(H7N9) or A(H5N1) virus.

(a) Mice were intranasally infected with A/Anhui/1/2013 (H7N9) and treatment was started immediately after virus inoculation (n = 5/group). The proinflammatory cytokines and chemokines, IL-6 and MCP-1, in the lungs at 1, 3 and 5 dpi were quantified. (b) Mice were intranasally infected with A/Hong Kong/483/1997 (H5N1) and treatment was started immediately after virus inoculation (n = 5/group). The IL-6 and MCP-1 in the lungs at 5 dpi were quantified. The bars represent the mean levels of IL-6 or MCP-1 + SD per group. Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily. Dunnett's multiple-comparison method was employed for comparison (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle, † $p < 0.05$, †† $p < 0.01$ compared with OSP at 5 mg/kg).

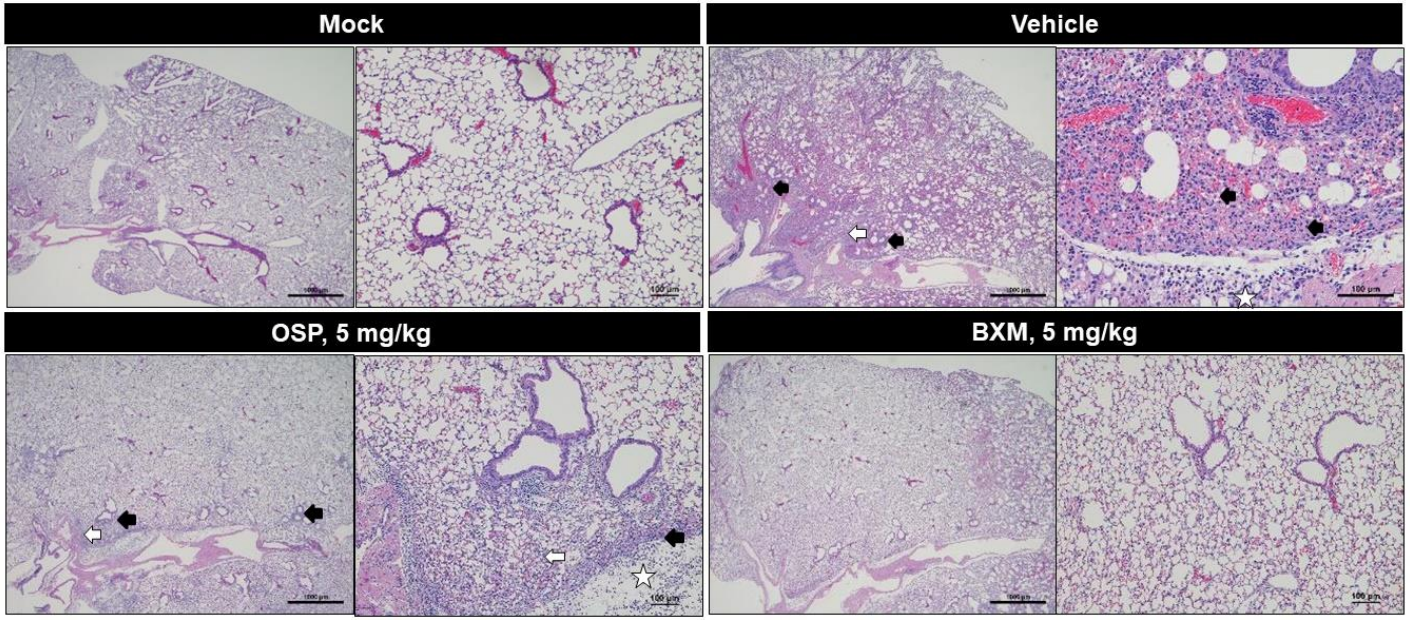

Figure 5. Prevention of inflammation in the lungs of mice infected with A(H5N1) virus following BXM treatment.

The mice were intranasally infected with A/Hong Kong/483/1997 (H5N1), and treatment was started immediately after virus inoculation. The lungs after each dosing were collected and fixed in a perfusion, containing 10% phosphate-buffered formalin. The formalin-fixed left lungs were then dissected, embedded in paraffin, and sectioned. Hematoxylin and eosin-stained sections, which were prepared for specimen and histopathological analyses, were subsequently analyzed. The left panels of each dosing represent wide fields (2×magnification of objective, scale bar: 1,000 μm), whereas the right panels of each dosing represent narrow fields (20×magnification of objective, scale bar: 100 μm). The black arrows indicate thickening and inflammatory cell infiltration of alveolar walls. The white arrows indicate inflammatory cell infiltration within the alveoli. The white stars indicate edema. Vehicle: 0.5 w/v% methylcellulose. Mock: the mice were inoculated with Dulbecco's phosphate-buffered saline and administered vehicle twice daily for 5 days.

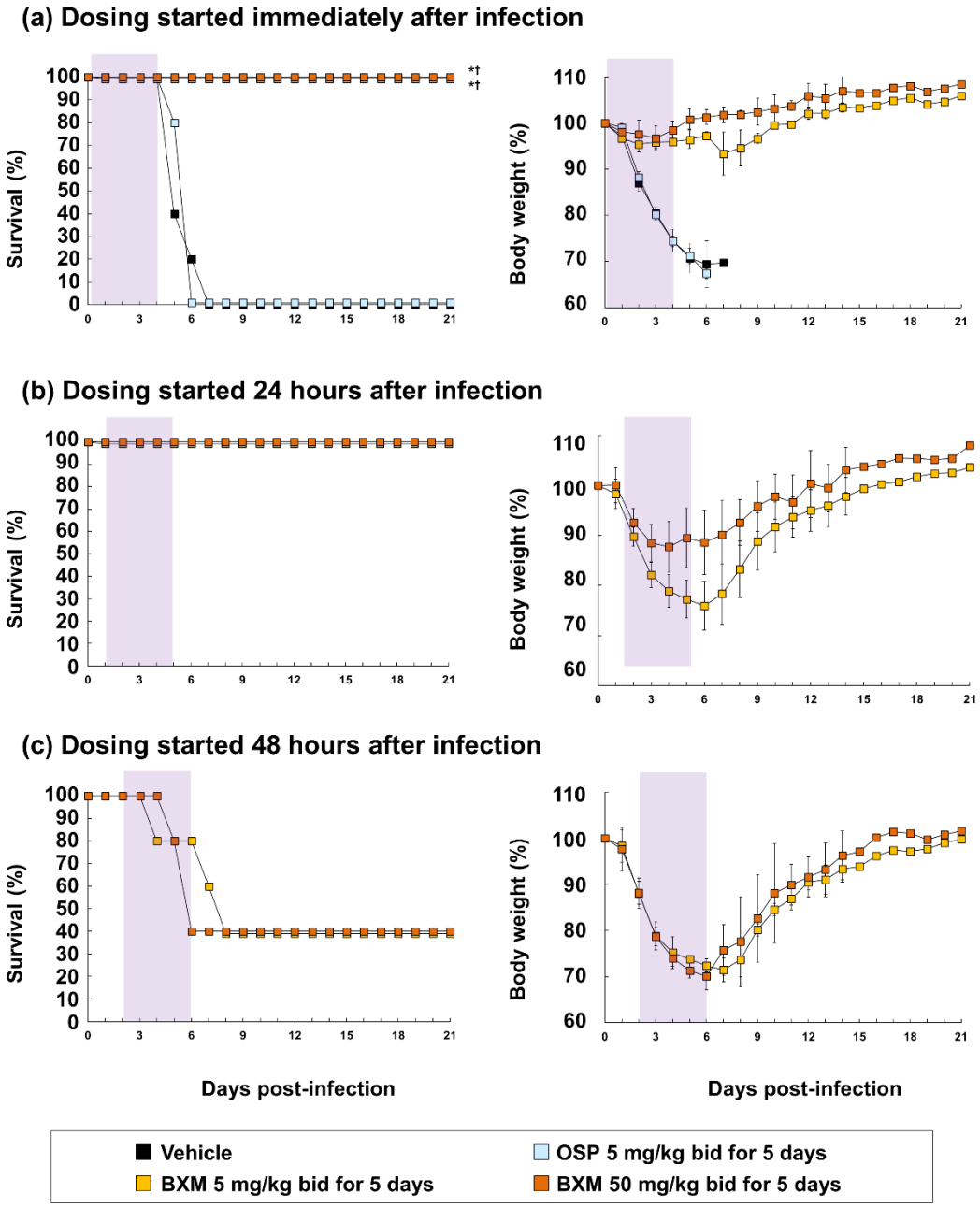

Figure 6. Effects of delayed treatment with BXM on A(H7N9) virus infection.

Mice were intranasally infected with A/Anhui/1/2013 (H7N9), and BXM treatment was started at (a) immediately, (b) 24 or (c) 48 hours after virus inoculation (n = 5/group). Vehicle (0.5 w/v% methylcellulose) or OSP treatment was started immediately after virus inoculation (n = 5/group). Survival and body weight loss were monitored by 21 dpi. The shaded area represents the treatment period. bid (bis in die): twice daily. The log-rank test was applied for comparison of the survival time between each group (*$p < 0.01$ compared to vehicle, †$p < 0.01$ compared to OSP at 5 mg/kg).

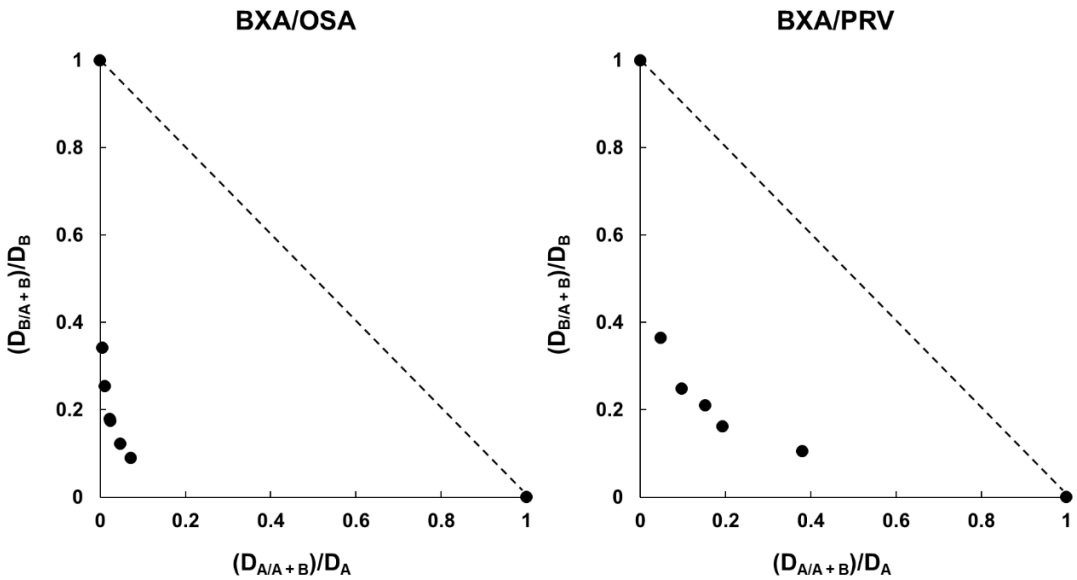

Figure 7. The isobologram plot of BXA combined with OSA or PRV.

The EC_{50} of each substance alone and at a fixed concentration were determined. $(D_{A/A+B})/D_A$ and $(D_{B/A+B})/D_B$ were plotted on the x and y-axes, respectively. D_A is the EC_{50} of substance A alone. D_B is the EC_{50} of substance B alone. $D_{A/A+B}$ is the concentration of substance A; given a 50% inhibition combined with substance B. $D_{B/A+B}$ is the concentration of substance B; given a 50% inhibition combined with substance A.

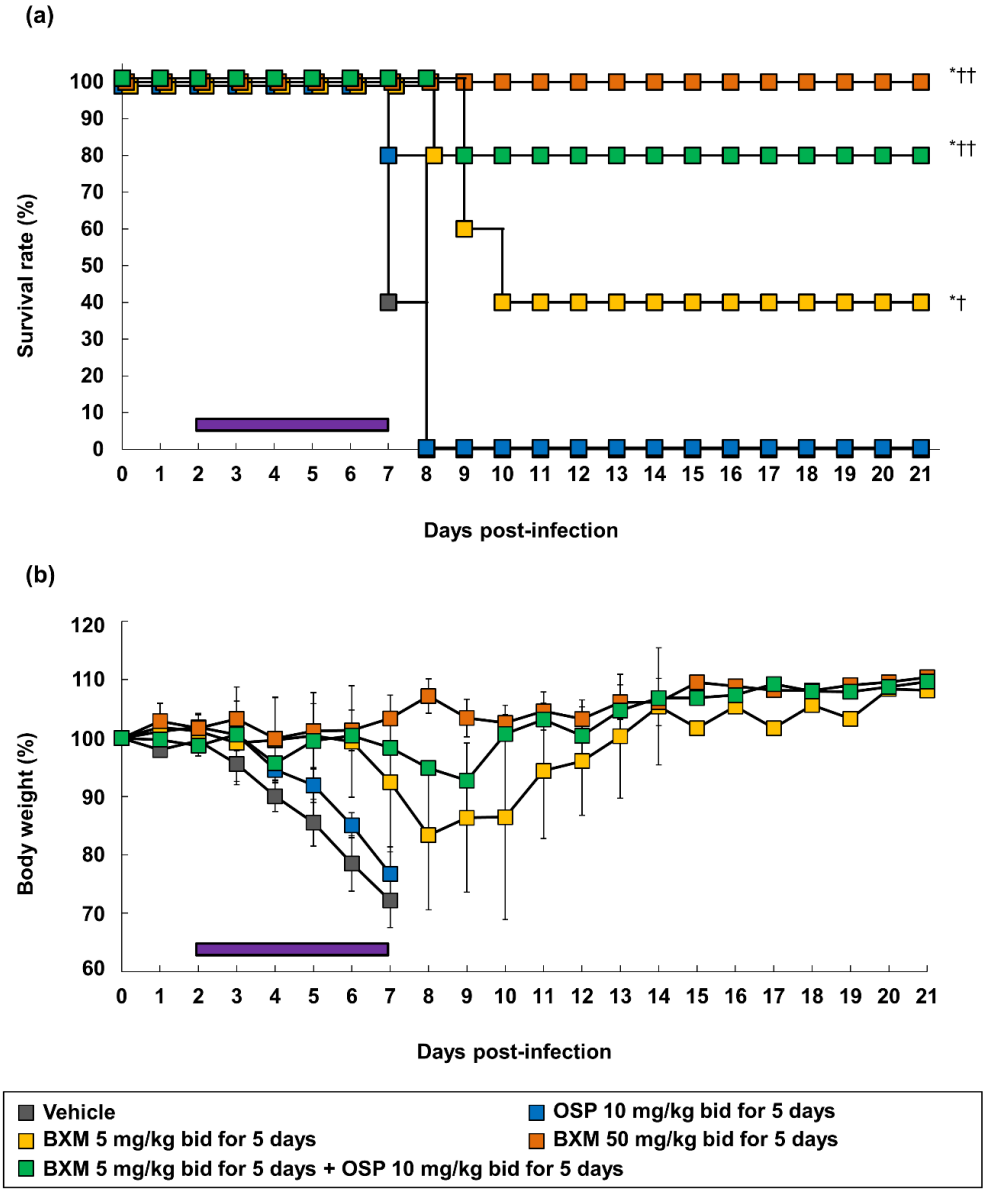

Figure 8. Effects of the delayed treatment with BXM, OSP or their combination on A(H5N1) virus infection in mice.

The mice were intranasally infected with A/Hong Kong/483/1997 (H5N1), and treatment was started 48 hours after virus inoculation. (a) Survival and (b) body weight loss were monitored throughout a 21-day period after the infection (n = 5/group). The purple lane represents the treatment period. Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily. The log-rank test was performed for comparing the survival times between each group (*$p < 0.01$ compared with the vehicle. †$p < 0.05$, ††$p < 0.01$ compared with OSP at 10 mg/kg).

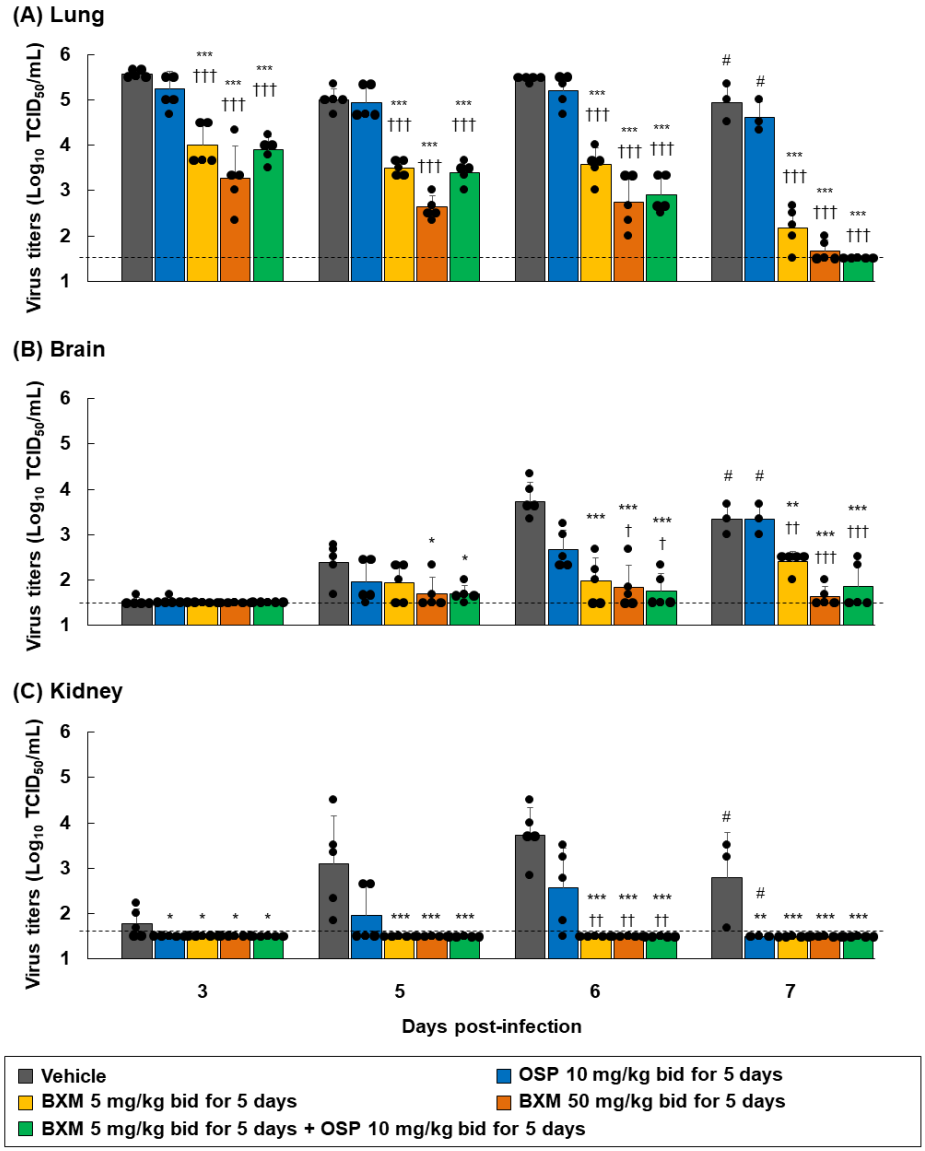

Figure 9. Inhibitory effects of delayed treatment of BXM, OSP, or their combination on the viral titers in the lungs, brains, and kidneys of mice infected with A(H5N1) virus.

The mice were intranasally infected with 75 $TCID_{50}$/mouse (31.3 MLD_{50}) of A/Hong Kong/483/1997 (H5N1), and treatment was started 48 hours after virus inoculation. Viral titers ($TCID_{50}$) in the (a) lungs, (b) brains, and (c) kidneys of mice at 3, 5, 6, and 7 dpi measured in MDCK cells (n = 5/group). The bars represent the mean viral titers + SD per group and individual viral titers for each sample are shown as black circles. The lower limit of quantification of the viral titer is indicated using a dotted line (1.5 $\log_{10}$ $TCID_{50}$/mL). Vehicle: 0.5 w/v% methylcellulose. bid (bis in die): twice daily. Dunnett's multiple-comparison test was conducted for a statistical comparison between the viral titers in each organ group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with vehicle, †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$ compared with OSP at 10 mg/kg). #, n = 3/group. Two of 5 mice in each group died at 7 dpi.

Table 3. The variants of PA region in viral RNAs derived from each tested avian influenza virus stock.

Amino acids differing from the reference sequence of each tested virus are highlighted in underlined. Each codon in parentheses corresponds to the above amino acid. Reference sequences of each tested virus were obtained from NCBI or GISAID. Each PA nucleotide sequence of all samples except above was completely matched with that of the reference (the parent virus). Call: base calling. -: PA nucleotide sequence was completely matched with that of the reference sequence. #: Sequence analysis was not performed because the sequence had already been determined. N.T.: not tested.

Strain name	Accession number	Nucleic acid				Amino acid			
		Position	Reference	Call	Minor peak	Position	Reference	Call	Minor peak
A/yellow-billed pintail/Chile/1/2012 (H1N1)	CY207202	97	A	A	T	33	N (AAT)	N (AAT)	Y (TAT)
A/red-fronted coot/Chile/5/2013 (H3N6)	CY207027	294	T	T	C	98	T (ACT)	T (ACT)	T (ACC)
A/pekin duck/California/P30/2006 (H4N2)	CY053826	-	-	-	-	-	-	-	-
A/shorebird/Delaware Bay/309/2008 (H4N6)	CY126603	111	A	A	C	37	A (GCA)	A (GCA)	A (GCC)
A/duck/Hokkaido/WZ20/2014 (H5N2)#	LC042072	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
A/ruddy turnstone/Delaware Bay/136/2007 (H6N1)	CY127788	-	-	-	-	-	-	-	-
A/duck/Yamagata/061004/2014 (H6N6)#	LC042080	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
A/laughing gull/Delaware Bay/50/2006 (H7N3)	CY095653	-	-	-	-	-	-	-	-

Table 3. (continued)

Strain name	Accession number	Nucleic acid				Amino acid			
		Position	Reference	Call	Minor peak	Position	Reference	Call	Minor peak
A/yellow-billed pintail/Chile/10/2014 (H7N3)	CY207226	87	A	A	G	29	K (AAA)	K (AAA)	K (AAG)
A/yellow-billed teal/Chile/9/2013 (H7N6)	CY207034	-	-	-	-	-	-	-	-
A/chicken/Netherlands/2586/2003 (H7N7)	AB438940	-	-	-	-	-	-	-	-
A/chicken/Vietnam/HU1-381/2014 (H9N2)[#]	LC069901	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
A/shorebird/Delaware Bay/139/2009 (H10N7)	CY137799	-	-	-	-	-	-	-	-
A/shorebird/Delaware Bay/549/2009 (H11N1)	CY127892	111	A	A	C	37	A (GCA)	A (GCA)	A (GCC)

Table 4. The variants of PA region in viral RNAs derived from each tested swine influenza virus stock.

The legends were referred in Table 3.

Strain name	Accession Number	Nucleic acid				Amino acid			
		Position	Reference	Call	Minor peak	Position	Reference	Call	Minor peak
A/swine/Iowa/15/1930 (H1N1)	M26076	75	A	G	-	25	G (GG<u>A</u>)	G (GG<u>G</u>)	-
		134	C	G	-	45	S (T<u>C</u>C)	C (T<u>G</u>C)	-
		172	G	A	-	58	G (<u>G</u>GC)	S (<u>A</u>GC)	-
		253	G	A	-	85	A (<u>G</u>CA)	T (<u>A</u>CA)	-
		366	T	A	-	122	V (GT<u>T</u>)	E (GA<u>A</u>)	-
		435	T	C	-	145	I (AT<u>T</u>)	I (AT<u>C</u>)	-
		519	C	T	-	173	T (AC<u>C</u>)	T (AC<u>T</u>)	-
		576	C	T	-	192	R (CG<u>C</u>)	R (CG<u>T</u>)	-

Table 4. (continued)

Strain name	Accession number	Nucleic acid				Amino acid			
		Position	Reference	Call	Minor peak	Position	Reference	Call	Minor peak
A/swine/Kagoshima/23/2012 (H1N1)	AB910570	-	-	-	-	-	-	-	-
A/swine/Okinawa/2/2005 (H1N1)	AB573799	-	-	-	-	-	-	-	-
A/swine/Ratchaburi/2000 (H1N1)[#]	AB434287	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
A/swine/Miyagi/5/2003 (H1N2)	LC431443	-	-	-	-	-	-	-	-
A/swine/Miyazaki/1/2006 (H1N2)	AB441175	-	-	-	-	-	-	-	-
A/swine/Missouri/2124514/2006 (H2N3)	EU258940	-	-	-	-	-	-	-	-
A/swine/Chachoengsao/2002 (H3N2)	AB571802	-	-	-	-	-	-	-	-
A/swine/Hong Kong/81/1978 (H3N2)	LC431427	87	A	A	T	29	K (AAA)	K (AAA)	N (AAT)
		180	A	A	C	60	S (TCA)	S (TCA)	S (TCC)
A/swine/Obihiro/10/1985 (H3N2)	AB573672	-	-	-	-	-	-	-	-
A/swine/Tochigi/14/2013 (H3N2)	AB914507	-	-	-	-	-	-	-	-
A/swine/Yokohama/aq114/2011 (H3N2)	AB741022	-	-	-	-	-	-	-	-
A/swine/Hong Kong/9/1998 (H9N2)	KX879588	-	-	-	-	-	-	-	-
A/swine/Hong Kong/10/1998 (H9N2)	EPI2077974	-	-	-	-	-	-	-	-

Table 5. Susceptibility of recombinant A/Hong Kong/483/97 (H5N1) viruses harboring PA amino acid substitutions to BXA, OSA and FPV.

Data represent the mean and SD from three independent experiments. Fold change was calculated by dividing the mean EC_{90} of each tested virus to the mean EC_{90} of WT virus. [a]Virus generated by reverse genetics.

	EC_{90} (nmol/L)								
	BXA		Fold	OSA		Fold	FPV		Fold
Strain name	Mean	SD	change	Mean	SD	change	Mean	SD	change
A/Hong Kong/483/97 (H5N1)[a]	1.1	0.5	1.0	17.3	6.3	1.0	10,409.7	1,826.6	1.0
A/Hong Kong/483/97 PA/I38F (H5N1)[a]	26.5	13.9	24.0	29.2	13.4	1.7	16,252.7	11,840.9	1.6
A/Hong Kong/483/97 PA/I38M (H5N1)[a]	17.1	4.7	15.5	30.3	11.7	1.7	12,698.5	7,518.7	1.2
A/Hong Kong/483/97 PA/I38T (H5N1)[a]	53.3	28.6	48.2	21.6	17.7	1.2	12,699.6	4,722.9	1.2

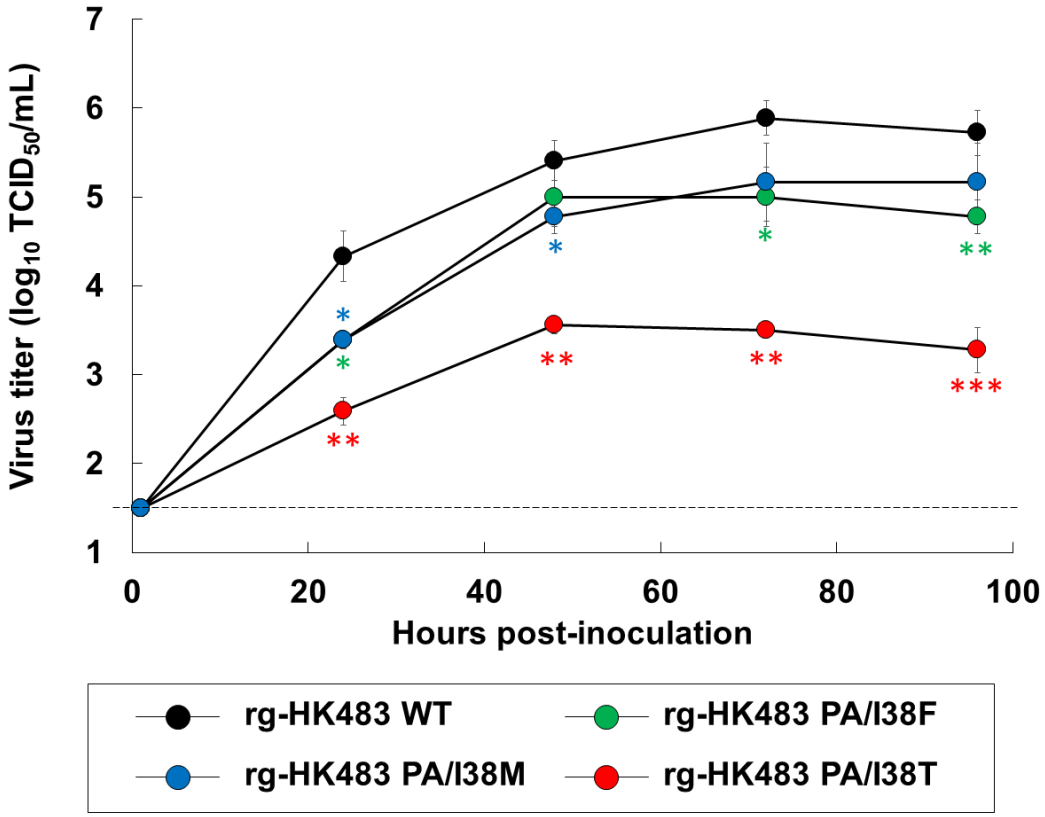

Figure 10. ***In vitro*** **replicative fitness of recombinant A/Hong Kong/483/97 (H5N1) viruses.** MDCK cells were infected with recombinant viruses at 100 $TCID_{50}$/well. Supernatants were harvested at the indicated time points, and the mean viral titers ± SD of triplicate wells were determined as $TCID_{50}$/mL using MDCK cells. The lower limit of quantification (1.5 $\log_{10}$ $TCID_{50}$/mL) of the viral titer is indicated by a dashed line. HK483: A/Hong/Kong/483/97 (H5N1). WT: wild type. Welch's *t*-test was conducted for statistical comparisons of titers between the WT virus and viruses with PA/I38F, M, and T substitutions at each time-point (*p< 0.05, **p< 0.01, ***p< 0.001 compared to WT virus).

Table 6. The EC_{90} values of BXA and FPV against the tested avian influenza viruses in yield reduction assays using MDCK cells.

Data represent the mean and SD of three independent experiments. The median EC_{90} values of BXA and FPV were 1.6 and 30,433.5 nmol/L, respectively.

*the viruses harboring PA amino acid polymorphisms (described in Table 8 in detail).

		EC_{90} (nmol/L)			
		BXA		FPV	
Strain name	Country	Mean	SD	Mean	SD
A/yellow-billed pintail/Chile/1/2012 (H1N1)	Chile	2.1	2.1	31,205.3	28,039.2
A/red-fronted coot/Chile/5/2013 (H3N6)	Chile	2.0	1.1	23,846.5	17,398.6
A/pekin duck/California/P30/2006 (H4N2)	US	2.1	1.2	8,459.1	3,869.7
A/shorebird/Delaware Bay/309/2008 (H4N6)	US	1.1	0.9	29,661.6	32,384.2
A/duck/Hokkaido/WZ20/2014 (H5N2)	Japan	0.9	0.8	14,931.3	11,108.6
A/ruddy turnstone/Delaware Bay/136/2007 (H6N1)	US	0.8	0.7	6,627.2	3,008.5
A/duck/Yamagata/061004/2014 (H6N6)	Japan	2.7	2.6	18,912.6	11,886.1
A/laughing gull/Delaware Bay/50/2006 (H7N3)	US	0.6	0.3	13,179.8	2,983
A/yellow-billed pintail/Chile/10/2014 (H7N3)	Chile	2.6	2.0	71,284.8	71,632.8
A/yellow-billed teal/Chile/9/2013 (H7N6)	Chile	2.3	1.1	66,809.9	57,540.6
A/chicken/Netherlands/2586/2003 (H7N7)	Netherlands	1.7	0.3	127,541.1	92,368.6
A/chicken/Vietnam/HU1-381/2014 (H9N2)*	Vietnam	0.8	0.5	62,342.3	58,578.3
A/shorebird/Delaware Bay/139/2009 (H10N7)	US	1.5	0.7	35,234	15,776.7
A/shorebird/Delaware Bay/549/2009 (H11N1)	US	1.3	0.4	44,721.8	39,518.3

Table 7. The EC_{90} values of BXA and FPV against the tested swine influenza viruses in yield reduction assays using MDCK cells.

Data represent the mean and SD of three independent experiments. The median EC_{90} values of BXA and FPV were 1.6 and 13,957.1 nmol/L, respectively.

*the viruses harboring PA amino acid polymorphisms (described in Table 8 in detail).

		EC_{90} (nmol/L)			
		BXA		FPV	
Strain name	Country	Mean	SD	Mean	SD
A/swine/Iowa/15/1930 (H1N1)	US	2.8	1.6	6,907.6	1,888.2
A/swine/Kagoshima/23/2012 (H1N1)	Japan	1.5	0.8	26,428.2	16,669.8
A/swine/Okinawa/2/2005 (H1N1)*	Japan	1.8	1.2	36,110.5	36,400
A/swine/Ratchaburi/2000 (H1N1)*	Thailand	3.6	1.8	23,645.9	34,236
A/swine/Miyagi/5/2003 (H1N2)*	Japan	3.1	3.0	9,954.1	5,718.4
A/swine/Miyazaki/1/2006 (H1N2)*	Japan	1.2	0.4	13,480.9	12,250.7
A/swine/Missouri/2124514/2006 (H2N3)	US	2.0	1.0	8,822.4	4,888.9
A/swine/Chachoengsao/2002 (H3N2)*	Thailand	3.5	2.2	13,363.6	10,734.1
A/swine/Hong Kong/81/1978 (H3N2)	China	1.3	1.1	14,826.5	11,814.6
A/swine/Obihiro/10/1985 (H3N2)	Japan	3.3	1.7	20,563.6	8,091.6
A/swine/Tochigi/14/2013 (H3N2)	Japan	1.1	0.5	13,386.4	13,356.3
A/swine/Yokohama/aq114/2011 (H3N2)	Japan	1.1	0.8	16,773.1	17,235.5
A/swine/Hong Kong/9/1998 (H9N2)	China	1.0	0.6	12,296.8	6,689.8
A/swine/Hong Kong/10/1998 (H9N2)	China	0.6	0.1	14,433.2	12,669.8

Table 8. Amino acid polymorphisms in the BXA binding domain of PA from avian and swine influenza viruses.

[a]Amino acids are referred as subscribed in Table 2. Amino acids differing from the consensus sequence of human IAVs are highlighted in bold and underlined. [b]Numbers shown in parentheses represent the frequency (%) of the most common variants among PA sequences from the analyzed viruses (total: 41,537). [c]The variants were as follows: V (0.41%), L (0.02%), T (0.02%), and M (0.01%).

Strain name	PA amino acid position[a]												
	20	23	24	34	37	38	41	80	108	119	130	134	199
A/chicken/Vietnam/HU1-381/2014 (H9N2)	A	E	Y	K	**S**	I	H	E	D	E	Y	K	E
A/swine/Okinawa/2/2005 (H1N1)	**T**	E	Y	K	A	I	H	E	D	E	Y	K	E
A/swine/Miyagi/5/2003 (H1N2)	**T**	E	Y	K	A	I	H	E	D	E	Y	K	E
A/swine/Miyazaki/1/2006 (H1N2)	**T**	E	Y	K	A	I	H	E	D	E	Y	K	E
A/swine/Ratchaburi/2000 (H1N1)	A	E	**H**	K	A	I	H	E	D	E	Y	K	E
A/swine/Chachoengsao/2002 (H3N2)	A	E	**H**	K	A	I	H	E	D	E	Y	K	E
Other viruses evaluated	A	E	Y	K	A	I	H	E	D	E	Y	K	E
Human, avian, and swine IAVs isolated[b]	A (94.7) T (5.2)	E (100)	Y (98.0) H (2.0)	K (99.9)	A (92.5) S (7.4)	I[c] (99.5)	H (100)	E (100)	D (100)	E (100)	Y (100)	K (100)	E (99.5)

Conclusion

IAVs cause significant symptoms in humans through seasonal epidemics. Sometimes, influenza pandemics arise irregularly because IAVs undergo constant reassortment in wild hosts, leading to the permanent possibility of the emergence of novel strains. Sporadic direct transmission of IAVs from animals to humans also poses a significant threat to human health with high morbidity and mortality. Any subtype of IAVs could possess the potential to infect humans in the future; therefore, the preparedness with broad-spectrum antiviral drugs is important for pandemic prophylaxis and treatment. BXA exhibited a broad spectrum against influenza A and B viruses harboring several subtypes including zoonotic strains. However, the efficacy of BXM, a prodrug form of BXA, on animal-derived IAVs such as A(H7N9) and A(H5N1) viruses has not been sufficiently verified. In addition, the characterization of PA/I38 substitutions known as a marker of reduced BXA susceptibility in seasonal influenza strains and PA polymorphisms involved in BXA susceptibility in zoonotic strains have not been fully elucidated. Therefore, the efficacy of BXM against animal-derived IAVs and PA amino acid polymorphisms involved in BXA susceptibility in zoonotic strains were evaluated in this study.

In Chapter I, the broad-spectrum efficacy of BXA against various animal-derived A(H7N9) and A(H5N1) viruses, including HPAIVs isolated from worldwide, including the strains harboring NAI-resistance mutations, were demonstrated in MDCK cells. The inhibitory efficacy of BXA was also higher than that of OSA or FPV. Moreover, BXM strongly inhibited A(H7N9) and A(H5N1) virus replication in the lungs and extra respiratory organs of mice. While inhibitory effect of OSP in mice lungs diminished with the passage of days after virus infection, the effect of BXM was sustained. BXM administration also prevented lung inflammation and improved mortality in an A(H7N9) and A(H5N1) virus-infected mice. BXM also exhibited a wide treatment window as both monotherapy and combination therapy with OSP.

In Chapter II, the characterization of PA/I38 variants of A(H5N1) was performed in MDCK cells. As a result, recombinant A(H5N1) viruses harboring PA/I38T, F and M were less susceptible to BXA, similar to seasonal IAVs. PA mutants also impaired replicative capacity in MDCK cells compared with the WT virus. All tested animal-derived strains were widely susceptible to BXA, regardless of isolation areas, subtypes, and dates of isolation. Moreover, new PA amino acid polymorphisms as a new marker for BXA susceptibility were not found.

The outcomes of these researches will contribute to the usage of BXM as one of

the therapies when the infection of the animal-derived influenza virus is confirmed in humans. Continuous monitoring of amino acid polymorphisms in the PA in naturally isolated animal-derived IAVs will remain important. Moreover, further analysis of animal-derived influenza virus strains harboring BXA resistance mutations and their characterization in animal models will be needed. This will contribute to increasing knowledge for pandemic countermeasures.

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Summary in Japanese（和文要旨）

インフルエンザは、インフルエンザウイルスの感染に起因するヒト及び動物の呼吸器感染症で、不定期にヒトで世界的大流行 (パンデミック) を繰り返している。それらは全て鳥とヒト、或いは鳥とブタ、ヒトの間での遺伝子再集合により生じたウイルスが原因である。また、H5N1 や H7N9 等、様々な亜型の鳥インフルエンザウイルスが散発的にヒトへ直接感染する事例も繰り返し報告されている。今後も全ての亜型のウイルス株がヒトに感染する可能性が考えられ、広域な有効性を示す予防・治療薬の備えが重要である。インフルエンザの治療には、罹患早期に抗インフルエンザ薬を投与することが重要である。米国疾病予防管理センターは、鳥インフルエンザウイルス感染時に、ヒトに対して抗ウイルス薬としてノイラミニダーゼ阻害薬 (NAI) の投与を推奨している。しかし、季節性インフルエンザウイルスで検出される薬剤耐性変異［NA/H275Y や NA/R292K (N2 ナンバリングで定義)］と同じ変異が鳥インフルエンザウイルス感染患者の治療中に検出される事例や、上記変異を保有する株が自然界で検出されており、治療の選択肢拡充が重要である。

バロキサビルマルボキシル (BXM) は、A 型及び B 型インフルエンザウイルス感染症の治療及び予防を目的に製造承認された低分子化合物である。本薬はプロドラッグであり、生体内で活性体であるバロキサビル酸 (BXA) に変換され、それがインフルエンザウイルスの PA タンパク質が保有するキャップ依存性エンドヌクレアーゼ活性を選択的に阻害し、ウイルスゲノムの転写過程を抑制する。PA タンパク質のアミノ酸配列の相同性は亜型によらず高く、BXA は A 型、B 型季節性インフルエンザウイルス株や、H5 や H7 亜型等の動物由来株を含む様々なウイルス株に対して有効性を示す。しかし、NAI 耐性変異を保有した動物由来株の BXA 感受性や、*in vivo* モデルでの動物由来株に対する BXM の有効性に関する情報は少なく、動物由来株に感染した患者に対する治療法の選択肢拡充のための知見の収集は重要である。また臨床では、PA タンパク質のアミノ酸 38 位のイソロイシン (PA/I38) の変異に代表される、BXA 感受性の低下に関わるアミノ酸変異が報告されている。しかし、動物由来株における PA/I38 変異の性状解析は不十分であり、BXA 感受性低下に関わる PA タンパク質のアミノ酸多型に関する知見も少ない。そこで本研究では、種々の動物由来株を用いて、BXM の有効性に関する *in vitro* 及び *in vivo* 試験を実施した。

第 I 章では、H5 及び H7 亜型株に対する BXM の有効性を検証した。その結果、近年国内で分離された株を含む複数の H5 及び H7 亜型高病原性鳥インフルエンザウイルスや、様々な NAI 耐性変異を導入して作出した組換え A/Hong Kong/483/1997 (H5N1、HK483) 株及び A/Anhui/1/2013 (H7N9、Anhui1) 株

が、BXA 感受性であることがわかった。またその阻害活性は、オセルタミビルやファビピラビルのそれらよりも高かった。なお、PA タンパク質のアミノ酸多型解析から、多くの H7 亜型株は PA/A37S 多型を有していた。本多型は、BXA が結合する PA タンパク質のエンドヌクレアーゼドメインの活性中心付近に位置しているが、BXA 感受性は変化しなかった。次に、Anhui1 株及び HK483 株をマウスに経鼻感染させ、感染直後より BXM 或いはリン酸オセルタミビル (OSP) を、5 mg/kg 或いは 50 mg/kg の用量で、1 日 2 回 5 日間経口投与した。その結果、BXM は感染翌日の肺内ウイルス増殖を OSP より強力に抑制し、以降の肺内ウイルス負荷を OSP より低く維持し続けた。なお、肺内から回収されたウイルスの PA タンパク質にはアミノ酸置換等の変異は確認されなかった。また BXM 投与によって、ウイルス感染に伴う肺組織の炎症が抑制され、致死抑制効果が認められた。HK483 株をマウスに感染させると、脳や腎臓からもウイルスが検出されるが、BXM 投与によって、それらが抑制された。さらに、Anhui1 或いは HK483 株の感染 24、48 時間後より BXM を投与した場合でも、致死抑制効果が認められた。また HK483 感染モデルで BXM と OSP (それぞれ 5 mg/kg、10 mg/kg を 1 日 2 回 5 日間) を併用投与することで、各化合物を単剤で投与するよりも優れた致死抑制効果が認められた。以上より、BXM は H5 及び H7 亜型インフルエンザウイルス感染時の治療オプションとして有用であることが示唆された。

第Ⅱ章では、PA/I38 に変異を有する動物由来株の性状解析のため、PA/I38F、PA/I38M、PA/I38T 変異を導入した組換え HK483 株をそれぞれ作出し、それらウイルスの BXA 感受性や MDCK 細胞における増殖性を検証した。また、世界各地で様々な年代に鳥とブタから分離されたウイルス株の BXA 感受性と、PA タンパク質のアミノ酸多型を解析した。その結果、各 PA/I38 変異を導入した組換え HK483 株の BXA 感受性が野生型株のそれより数十倍低下した。また各変異体の MDCK 細胞における増殖性が野生型株のそれより低下し、PA/I38T 変異を保有するウイルス株で特に顕著であった。なお、BXA とは異なる作用機序を有するオセルタミビル及びファビピラビルの感受性は、野生型株のそれらと同程度であった。次に、世界各地で様々な年代に分離された計 28 株の鳥とブタ分離株の BXA 感受性を調べたところ、いずれの株も BXA 感受性であることがわかった。また、過去 10 年間に分離された A 型インフルエンザウイルス株に認められる PA タンパク質上のアミノ酸多型をデータベース上で確認したところ、99%以上の株で PA/I38 が保存されている一方、PA/A20T、Y24H、A37S 等の多型が全株の 1%以上に認められた。なお、今回評価した株の一部に上記多型が認められたが、いずれも BXA 感受性は低下しなかった。以上の結果から、動物由来インフルエンザウイルスにおいても、PA/I38 が BXA の耐性変異マーカーとして有

用である可能性が示唆された。

以上より、第 I 章では、国内外で分離された高病原性鳥インフルエンザウイルスや NAI 耐性変異を導入した組換えウイルスを含む種々の H5 及び H7 亜型株に対する BXM の有効性が明らかとなった。本結果は、ヒトで動物由来インフルエンザウイルスの感染が確認された際の治療法の一つとして BXM が有用である事を示唆している。また第II章では、季節性インフルエンザウイルスの BXA 耐性変異マーカーとして知られる PA/I38 変異が動物由来株に導入された場合の *in vitro* における性状の一端が明らかとなった。また世界各地で様々な年代に分離された動物由来株が広く BXA に対して感受性を示し、BXA 感受性を低下させる新たな多型は見つからなかった。今後も、自然界で分離される動物由来株における PA タンパク質のアミノ酸多型の継続的なモニターや、BXA の耐性変異を有する動物由来株の更なる性状解析が重要である。本研究で得られた知見が、将来発生しうるインフルエンザパンデミックに活用されることが期待される。