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Authors: Miki, Mariko, Obara, Ryo Daniel, Nishimura, Kyohei, Shishido, Takao, Ikenaka, Yoshinori, et al.

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FOUR-WEEK ORAL ADMINISTRATION OF BALOXAVIR MARBOXIL AS AN ANTI-INFLUENZA VIRUS DRUG SHOWS NO TOXICITY IN CHICKENS

Mariko Miki, DVM, Ryo Daniel Obara, DVM, Kyohei Nishimura, PhD, Takao Shishido, PhD, Yoshinori Ikenaka, PhD, Ryoko Oka, Kenji Sato, MS, Shouta M.M. Nakayama, DVM, PhD, Takashi Kimura, DVM, PhD, Atsushi Kobayashi, DVM, PhD, Keisuke Aoshima, DVM, PhD, Keisuke Saito, DVM, PhD, Takahiro Hiono, DVM, PhD, Norikazu Isoda, DVM, PhD, and Yoshihiro Sakoda, DVM, PhD

Abstract: High pathogenicity avian influenza is an acute zoonotic disease with high mortality in birds caused by a high pathogenicity avian influenza virus (HPAIV). Recently, HPAIV has rapidly spread worldwide and has killed many wild birds, including endangered species. Baloxavir marboxil (BXM), an anti-influenza agent used for humans, was reported to reduce mortality and virus secretion from HPAIV-infected chickens (*Gallus domesticus*, order Galliformes) at a dosage of ≥2.5 mg/kg when administered simultaneously with viral challenge. Application of this treatment to endangered birds requires further information on potential avian-specific toxicity caused by repeated exposure to BXM over the long term. To obtain information of potential avian-specific toxicity, a 4-wk oral repeated-dose study of BXM was conducted in chickens ($n = 6$ or 7 per group), which are commonly used as laboratory avian species. The study was conducted in reference to the human pharmaceutical guidelines for nonclinical repeated-dose drug toxicity studies to evaluate systemic toxicity and exposure. No adverse changes were observed in any organs examined, and dose proportional increases in systemic exposure to active pharmaceutical ingredients were noted from 12.5 to 62.5 mg/kg per day. BXM showed no toxicity to chickens at doses of up to 62.5 mg/kg per day, at which systemic exposure was approximately 71 times higher than systemic exposure at 2.5 mg/kg, the reported efficacious dosage amount, in HPAIV-infected chickens. These results also suggest that BXM could be considered safe for treating HPAIV-infected endangered birds due to its high safety margin compared with the efficacy dose. The data in this study could contribute to the preservation of endangered birds by using BXM as a means of protecting biodiversity.

INTRODUCTION

High pathogenicity avian influenza (HPAI), an acute zoonotic disease caused by high pathogenicity avian influenza virus (HPAIV) infection, is highly infectious and lethal, especially in birds.[26] The incubation period of HPAIV in birds reportedly ranges from 1 to 7 d for individual birds and 14 d for flocks in which an HPAIV-infected bird is found, and it depends on the species, age, and virus genotype.[39,47] Clinical symptoms progress rapidly after HPAIV infection, especially for Galliformes species, including chickens (*Gallus domesticus*).[1,5,18] Most HPAIV-infected galliforms die within 2–7 d.[6,18,26,37]

The number of outbreaks of HPAI among wild birds is increasing worldwide, posing a serious animal health problem. The number of cases of H5

From the Shionogi & Co, Ltd, 1-1, Futaba-cho 3-chome, Toyonaka 561-0825, Japan (Miki, Obara, Nishimura, Shishido, Oka, Sato); Laboratory of Microbiology, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan (Hiono, Isoda, Sakoda); Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan (Ikenaka, Nakayama); Translational Research Unit, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan (Ikenaka); Laboratory of Comparative Pathology, Department of Clinical Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan (Kimura, Kobayashi, Aoshima); One Health Research Center, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan (Ikenaka, Hiono, Isoda, Sakoda); Institute for Raptor Biomedicine Japan, 2-2101 Hokuto, Kushiro 084-0922, Japan (Saito); International Collaboration Unit, International Institute for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10, Kita-Ku, Sapporo 001-0020, Japan (Hiono, Isoda, Sakoda); Hokkaido University Institute for Vaccine Research and Development, Hokkaido University, Kita 21, Nishi 11, Kita-ku, Sapporo 001-0021, Japan (Sakoda); Water Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom 2531, South Africa (Ikenaka); and Biomedical Sciences Department, School of Veterinary Medicine, The University of Zambia, P.O. Box 32379, Lusaka, Zambia (Nakayama). Present address (Kobayashi): Department of Biomedical Models, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4, Sakamoto, Nagasaki 852-8523, Japan. Correspondence should be directed to Dr. Miki (mariko.miki@shionogi.co.jp).

HPAIV infection in wild birds exceeded the number in poultry and domestic birds in 36 European countries in the 2021–2022 season.[7] Outbreaks of H5 HPAIV in wild birds have occurred in Asian countries from 2020[13,28] and in North American countries in 2022–2023.[48] Because infected wild birds can carry HPAIV to other regions of the world through long distance migration,[11] it seems that HPAIV transmission in wild bird populations and the occurrence of outbreaks of HPAI in birds have already become uncontrollable. HPAIV infections and deaths in endangered birds listed by the International Union for Conservation of Nature Red List have been reported.[17,38] Current data suggest that other endangered species are also at risk of HPAIV infection, and HPAIV could therefore accelerate the extinction of these endangered species, thereby posing a threat to biodiversity.[33,34]

Detection and culling are the basic control measures for HPAI in poultry worldwide; however, improved control measures that would protect HPIAV-infected endangered species could be invaluable. Biodiversity could be maintained by the Conservation of Endangered Avian Species.[3,33,46] This study was therefore conducted to determine optimal treatment for HPAIV-infected endangered avian species to preserve biodiversity.

In chickens infected with HPAIV, mortality and virus replication are reportedly reduced by a single oral administration of baloxavir marboxil (BXM), an anti-influenza agent for treatment and prophylaxis in humans,[29] when administered at a dose ranging from 2.5 to 62.5 mg/kg simultaneously with HPAIV challenge.[42] BXM selectively inhibits the activity of cap-dependent endonuclease, an influenza virus–specific enzyme in the polymerase acidic (PA) subunit of the viral RNA polymerase complex.[25] Cap-dependent endonuclease is essential for virus mRNA synthesis, which thereby inhibits influenza virus replication.[4] The pharmaceutical effect of BXM is initiated by metabolism of the prodrug form to baloxavir acid (BXA), the active form.[2] It has been reported that BXA shows broad antiviral potency against various types of influenza viruses, most likely because the structure of the active site of cap-dependent endonuclease in the PA subunits is similar among influenza viruses.[23] BXA exhibited similar *in vitro* antiviral activity against H5N1, H5N6, and H5N8 subtypes tested in comparison with human influenza A (H1N1 and H3N2) virus subtypes.[40] BXM treatment of mice infected with H5N1 or H7N9 HPAIV exhibited significantly reduced viral titers in tissues, which prevented the development of acute inflammation and reduced mortality.[40,41]

The safety of BXM in mammals has been confirmed by nonclinical studies, which confirmed the nonobservable adverse effect level (NOAEL) of 2,000 mg/kg per day in rats, 10 mg/kg per day in monkeys, and 100 mg/kg per day in rabbits.[29] However, birds exhibit anatomical differences from mammals, with variable gastrointestinal, lymphoid, and respiratory structures[9,19,21,24]; the presence of nucleated erythrocytes and heterophils as blood cells;[35] and physiological differences related to immunity and drug-metabolizing enzymes, such as in chickens.[19,38,44] In addition, birds and mammals exhibit clinical-pathological differences such as the sensitivity and specificity of alanine transaminase and bilirubin following liver injury, as seen in pigeons.[10,12] These differences between birds and mammals must be considered when evaluating the safety of BXM in birds. Confirming the safety of BXM in endangered avian species requires the use of bird models and protocols designed specifically for birds intended for BXM treatment. Moreover, it is important to note that avian taxa display numerous differences anatomically and metabolically, such as those between chickens, ostriches (*Struthio camelus*), mallards (*Anas platyrhynchos*), and owls (Strigiformes).[19,20,24,44]

Herein, a 4-wk oral repeated-dose toxicity study of BXM in chickens is described. A single-dosing toxicity study was conducted before the repeated-dose toxicity study for the purpose of dose selection. In addition to information regarding the efficacy of BXM, information regarding the safety of BXM in chickens will contribute to the conservation of biodiversity by reducing mortality in endangered birds infected with HPAIV. Developing a new treatment strategy for endangered birds could aid preservation efforts for endangered birds.

MATERIALS AND METHODS

Experiment 1: single oral administration toxicokinetics (TK) study of BXM in chickens (single-dosing TK study)

This study was conducted before the 4-wk toxicity study of BXM in chickens (see Experiment 2 section) to collect data for dose selection in the 4-wk toxicity study.

Test substance preparation: A 0.5 w/v% methylcellulose (MC) aqueous solution (0.5 w/v% MC, FUJIFILM Wako Pure Chemical Corp, Osaka 540-8605, Japan) was prepared as the vehicle. An appropriate amount of BXM (20-mg tablets, Shionogi & Co, Ltd, Osaka 540-8611, Japan) was

ground and suspended in the vehicle by using a mortar and pestle to prepare 1-, 5-, and 25 mg/ml formulations.

Study design: Two ($n = 2$) 10-wk-old Julia-Lite female chickens (Belbird Ltd, Chiba 286-0118, Japan) and six ($n = 6$) 10-wk-old male Julia chickens and six ($n = 6$) 10-wk-old female Julia chickens (Belbird Ltd) were used in this study. This study was conducted using young adult 10-wk-old chickens, with references to guidelines for the testing of chemicals in rodents and guidance for tolerance studies in the target animals of substances used in animal feed.[8,27] Three doses—2.5, 12.5, and 62.5 mg/kg—were set to obtain dose–response data. The formulations of BXM were orally administered to chickens (2.5 ml/kg; calculated from the latest body weight) by using a pipette, with dose escalation at approximately 1-wk washout intervals. The birds were observed daily for mortality and any clinical signs. After the final blood sampling, all of the birds were euthanized by IV injection of thiopental (150 mg/kg).

Examination: For the single-dosing TK study, the sampling points on each administration day were 1, 2, 8, 24, and 48 h after dosing. The concentrations of BXM and BXA were determined using liquid chromatography–tandem mass spectrometry. The below the limit of quantitation (BLQ) value for BXA was 0.004 μg/ml, and the value for BXM was 0.0002 μg/ml. The TK parameters maximum plasma concentration (C_{max}), area under the plasma concentration–time curve from 0 to 24 h ($AUC_{0–24h}$), determined using the linear trapezoidal method, and time to maximum plasma concentration (T_{max}) were calculated using PK plus software pharmacokinetic software, PKPlus (Northern Science Consulting Inc., Sapporo 060-0005, Japan). The area under the plasma concentration–time curve from 0 h to infinity (AUC_{inf}) was also simulated and calculated at 2.5 mg/kg by non-compartmental analysis. The AUC_{inf} value is equal to the area under the plasma concentration–time curve from 0 to time tau (the dosing interval) at the steady state (AUC_{τ}).[36]

Experiment 2: oral administration toxicity study of BXM in chickens (4-wk toxicity study)

Test substance preparation: MC and BXM formulations (0.5 w/v%) were prepared in the same manner as described for the single-dosing TK study.

Study design: Three groups of six or seven ($n = 6$ or 7 per group) 10-wk-old Julia-Lite female chickens (Belbird Ltd) were used in this study. BXM formulation was administered to chickens daily for 4 wk at a dosage of 0, 12.5, or 62.5 mg/kg per day. From the results of the preliminary single-dosing TK study, doses of 12.5 and 62.5 mg/kg per day were set to obtain dose–response data. Formulations of either BXM or vehicle were orally administered (2.5 ml/kg; calculated from the latest body weight) to the birds daily in the same manner as used in the single-dosing TK study.

Examination: Examination items were selected with reference to the guidelines for repeated-dose toxicity studies of drugs to evaluate systemic toxicity and exposures (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use 1998). For clinical observation, the birds were observed daily for mortality and any clinical signs. Body weight was measured for all birds two or three times per week. For measurement of food consumption, the birds were fed approximately 70 g/d per bird. The amount of leftover food (percent) the next morning was visually estimated using a poly beaker cup. Food consumption (grams per day) was calculated using the formula $70 \times (1 - \text{leftover amount [\%]}/100)$. Hematologic analyses were conducted for all birds on Days −1 and 27. Whole blood treated with EDTA-2K (Greiner Bio-One Co. Ltd., Frickenhausen 72636, Germany) was used to determine the RBC, WBC, Hct, and differential counts.[35,43] Plasma obtained from blood treated with 3.2% sodium citrate solution was analyzed using a semiautomated blood coagulation analyzer (CA-104, Sysmex Corp, Hyogo 651-0073, Japan) to determine the prothrombin time (PT), as described in a previous report.[31] Blood chemistry parameters were measured for all birds on Days −1 and 27. Plasma obtained from blood treated with heparin sodium was analyzed to measure aspartate aminotransferase (AST), bile acid (BA), creatine kinase, uric acid, glucose (Glu), phosphorus, calcium, total protein (T. Pro), albumin (Alb), globulin (Glob), potassium, and sodium by using a chemistry analyzer (VetScan® VS2, Avian Reptilian Profile Plus, Zoetis, Parsippany, NJ 07054, USA).[49] At the completion of the administration period (Day 29), all of the birds were euthanized by IV injection of thiopental (150 mg/kg) and necropsied for pathological examination. The abdominal cavity, brain (including cerebrum, cerebellum, optic lobe, brain stem), bursa of Fabricius, ileum, cecum, colon, crop, duodenum, femur (including bone marrow), eyes, heart, jejunum, kidney, liver (including gall bladder), lung, oral cavity, ovary, oviduct, pancreas, parathyroid, spleen, stomach, thymus, and any other abnormal organs were examined macroscopically in all birds. For histopathologic analysis, the above-mentioned organs,

Table 1. Mean plasma concentration, maximum plasma concentration (C_{max}), area under the plasma concentration–time curve from 0 to 24 h ($AUC_{0–24h}$), and time to maximum plasma concentration (T_{max}) of baloxavir acid (BXA, active form) in the single-dosing toxicokinetics study in Julia-Lite and Julia chickens (*Gallus domesticus*, Galliformes). Area under the plasma concentration–time curve from 0 h to infinity (AUC_{inf}) was also calculated only for 2.5 mg/kg in Julia-Lite chickens. The sampling times were 1, 2, 8, 24, and 48 h after dosing. Parameters were calculated using pharmacokinetics software, PK plus. Three doses (2.5, 12.5, and 62.5 mg/kg) were orally administered to two or six chickens with dose escalation at approximately 1-wk washout intervals at 10, 11, and 12 wk old at the administration day, respectively.

Dose (mg/kg)	Strain	Sex	No. of birds	Time after administration (h) Plasma concentration of BXA (μg/ml) 1	2	8	24	48	C_{max} (μg/ml)	$AUC_{0–24h}$/ AUC_{inf}[a] (μg · h/ml)	T_{max} (h)
2.5	Julia-Lite	Female	2	0.314	0.366	0.172	0.028	0.006	0.370	3.709/4.198	1.5
12.5	Julia-Lite	Female	2	1.538	1.818	0.775	0.158	0.027	1.819	17.700	2.0
62.5	Julia-Lite	Female	2	5.248	6.084	4.540	1.265	0.105	7.231	86.610	5.0
2.5	Julia	Female	6	0.126	0.342	0.151	0.025	0.004	0.387	3.184	5.0
12.5	Julia	Female	6	0.995	1.608	0.599	0.112	0.024	1.649	14.111	1.8
62.5	Julia	Female	6	3.229	6.380	4.221	0.939	0.068	6.961	79.498	4.0
2.5	Julia	Male	6	0.130	0.240	0.146	0.030	0.004	0.276	2.818	4.0
12.5	Julia	Male	6	0.783	1.208	0.533	0.129	0.022	1.387	11.906	1.8
62.5	Julia	Male	6	1.534	2.236	3.859	1.076	0.069	4.211	60.422	7.0

[a] AUC_{inf} was calculated only 2.5 mg/kg in Julia-Lite.

except the cavities, were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Three-micrometer-thick sections were then stained with H&E. For TK analyses, the plasma concentration of BXM in the dosing groups was determined on Days 14 and 28 in the same manner as used for the single-dosing TK study. Sampling points included before (approximately 24 h after the previous dosing) and 2, 8, and 24 h after dosing. TK analysis was using plasma samples from three or four birds in the BXM dosing group. The BLQ value for BXA and BXM was 0.24 and 0.0002 μg/ml, respectively.

Statistical analyses

The mean and SD of values for body weight, food consumption, hematology, and blood chemistry parameters were calculated and analyzed using SAS 9.2 software in Provantis (version 9.3.2.2; Instem)[14] to compare the test substance dosing groups with the vehicle control group. All statistical tests were two sided and carried out using Dunnett's test. When the P value was <0.05, the difference relative to the control group was regarded as statistically significant.

Ethical considerations

All animal experiments were conducted with the approval of the Institutional Animal and Committee of Hokkaido University (approval 21-0102) and performed under the guidelines of the Institutional Animal and Committee of Hokkaido University, certified by the Association for Assessment and Accreditation of Laboratory Animal Care International since 2007.

RESULTS

Experiment 1: single oral administration TK study of BXM in chickens (single-dosing TK study)

There were no BXM-related abnormalities in any of the birds in terms of clinical signs, including moribund state and death. In Julia-Lite and Julia chickens, the mean C_{max} and $AUC_{0–24h}$ values for BXA increased in a dose-dependent manner from 2.5 to 62.5 mg/kg and the values were much higher than those for BXM (Table 1; Supplemental Fig. S1). There were no sex or strain differences in systemic exposure. Based on these results, the TK data of this study were substituted to the 4-wk toxicity study on Day 1. The mean C_{max} and $AUC_{0–24h}$ values for BXM were much lower than the values for BXA (Supplemental Table S1). Moreover, the mean AUC_{inf} value for BXA simulated based on the calculated efficacy dose for inhibition of HPAIV replication, 2.5 mg/kg, was 4.198 μg·h/ml in female Julia-Lite chickens.

Experiment 2: oral administration toxicity study of BXM in chickens (4-wk toxicity study)

There were no signs of BXM-related adverse effects in clinical signs, body weight (Fig. 1), food consumption (Supplemental Fig. S2), hematology (Table 2), blood chemistry (Table 3), or pathological examinations.

In blood chemistry analyses (Table 3), on Day 27, the calcium level in the 62.5 mg/kg per day

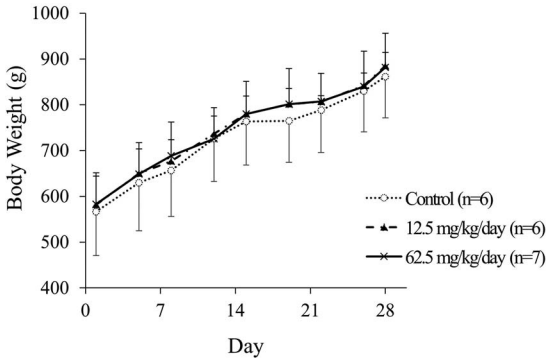

Figure 1. Change in body weight in the 4-wk toxicity study in six or seven per group female chickens (*Gallus domesticus*, Galliformes) at 10 wk old at the start day of administration. Daily administration of each dose was started on Day 1. Body weight of each chicken was measured on Days 1, 5, 8, 12, 15,19, 22, 26, and 28, and the average weight of the chickens in each group at each time point was calculated. Data represent the mean ± SD. There were no statistically significant differences between the control group and the BXM dosing groups.

dosing group was statistically lower than that of the control group ($P < 0.05$), whereas the Glu level in the 12.5 mg/kg per day dosing group was statistically lower than that of the control group ($P < 0.01$). On Day −1, the phosphorus level in the 12.5 and 62.5 mg/kg per day dosing groups was statistically lower than that of the control group ($P < 0.05$) and the Glu level in the 62.5 mg/kg per day dosing group was statistically higher than that of the control group ($P < 0.01$). In hematology (Table 2), percentages of heterophils and lymphocytes were statistically lower and higher, respectively, than these of the control group on Day −1 ($P < 0.01$).

In the TK analysis, the mean C_{max} and $AUC_{0–24h}$ values for BXA on Days 14 and 28 increased in a near dose-dependent manner from 12.5 to 62.5 mg/kg per day (Table 4; Supplemental Fig. S3). In terms of the effect of repeated dosing of BXA from Days 14 to 28, the mean C_{max} and $AUC_{0–24h}$ values on Days 14 and 28 were similar and the T_{max} value on Day 28 tended to be later than that on Day 14 (Table 4; Supplemental Figure S3). The mean C_{max} and $AUC_{0–24h}$ values for BXM were much lower than the values for BXA (Supplemental Table S2).

DISCUSSION

To obtain new and useful information regarding the safety of BXM in birds, a 4-wk repeated-dose toxicity study was conducted in chickens as a model avian species. This is the first study to provide data regarding avian-specific toxicity of BXM in chickens, an avian species used in laboratory studies and as a nonclinical model species. The criteria used to evaluate avian-specific toxicity in this study were selected in reference to the human pharmaceutical guidelines for repeated-dose toxicity studies,[16] also considering avian-specific characteristics in terms of organs and tissues and clinical pathological parameters. The duration of the study was set to 4 wk for two reasons: first, a 4-wk study would be the same duration as that of mammalian nonclinical toxicity studies, which are based on human pharmaceutical guidelines for medicines with an intended dosing period of <2 wk;[15] and second, because the duration of HPAIV shedding is reportedly no longer than 7 d,[18] it was presumed that the duration of BXM administration to endangered birds would be <2 wk.

The TK results indicate that systemic exposure to the prodrug (BXM) was much lower than the exposure to the active form (BXA) at all sampling times, suggesting that BXM is extensively

Table 2. Hematology data[a] on Days −1 and 27 for 0, 12.5, and 62.5 mg/kg per day dosing groups in six or seven female chickens (*Gallus domesticus*, Galliformes) at 10 wk old at the start day of administration in the 4-wk toxicity study.

Dose	Control		12.5 mg/kg per day		62.5 mg/kg per day	
Day	−1	27	−1	27	−1	27
No. of birds	6	6	6	6	7	7
RBC count ($\times 10^9$/ml)	3.31 ± 1.00	2.64 ± 0.42	3.24 ± 0.78	2.53 ± 0.17	2.88 ± 0.65	2.37 ± 0.33
Hct (%)	34.2 ± 6.1	30.3 ± 1.4	37.0 ± 10.5	29.5 ± 1.8	35.4 ± 8.5	28.6 ± 2.3
WBC count ($\times 10^6$/ml)	16.80 ± 3.81	17.20 ± 6.27	16.64 ± 10.11	15.58 ± 3.81	21.51 ± 9.53	14.21 ± 5.13
Heterophils (%)	44.5 ± 14.8	21.8 ± 12.2	33.7 ± 10.8	12.7 ± 5.9	17.1 ± 5.1**	15.6 ± 6.1
Lymphocytes (%)	47.5 ± 15.7	62.2 ± 20.2	57.0 ± 11.0	75.3 ± 6.8	75.0 ± 4.4**	71.0 ± 8.3
Monocytes (%)	6.5 ± 5.5	14.3 ± 9.1	6.7 ± 3.5	8.2 ± 4.4	6.4 ± 2.4	11.3 ± 5.9
Eosinophils (%)	0.8 ± 0.8	1.5 ± 1.4	2.0 ± 2.3	3.3 ± 1.5	1.4 ± 1.1	1.9 ± 2.1
Basophols (%)	0.7 ± 0.8	0.2 ± 0.4	0.7 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	0.3 ± 0.5
Prothrombin time (s)	11.7 ± 0.4	12.5 ± 3.4	11.9 ± 0.6	14.0 ± 1.1	11.5 ± 0.3	14.1 ± 2.3

[a] Values are mean ± SD. **$P < 0.01$ vs control.

Table 3. Blood chemistry data[a] on Days −1 and 27 for 0, 12.5, and 62.5 mg/kg per day dosing groups in six or seven female chickens (*Gallus domesticus*, Galliformes) at 10 wk old at the start day of administration in the 4-wk toxicity study.

Dose	Control		12.5 mg/kg per day		62.5 mg/kg per day	
Day	−1	27	−1	27	−1	27
No. of birds	6	6	6	6	7	7
Total protein (g/dl)	3.7 ± 0.5	4.2 ± 0.3	3.7 ± 0.2	4.1 ± 0.2	3.5 ± 0.3	3.9 ± 0.4
Albumin (g/dl)	2.5 ± 0.2	2.9 ± 0.1	2.5 ± 0.1	2.9 ± 0.1	2.4 ± 0.1	2.7 ± 0.2
Globulin (g/dl)	1.3 ± 0.4	1.3 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.3	1.2 ± 0.4
Creatine kinase (U/L)	1509 ± 425	1269 ± 198	1550 ± 370	1242 ± 119	1570 ± 495	1380 ± 342
Aspartate aminotransferase (U/L)	205 ± 58	179 ± 13	167 ± 7	168 ± 15	180 ± 8	171 ± 13
Bile acid (μmol/L)	<35[b]	<35[b]	<35[b]	<35[b]	<35[b]	<35[b]
Glucose (mg/dl)	247 ± 25	222 ± 6	231 ± 6	210 ± 7**	225 ± 8*	218 ± 7
Phosphorus (mg/dl)	4.6 ± 0.7	5.6 ± 0.3	5.9 ± 0.6*	5.5 ± 0.4	5.7 ± 0.7*	5.5 ± 0.4
Calcium (mg/dl)	10.9 ± 0.5	11.5 ± 0.4	11.0 ± 0.4	11.2 ± 0.3	10.8 ± 0.2	10.9 ± 0.3*
Sodium (mmol/L)	149 ± 2	150 ± 2	149 ± 3	149 ± 1	150 ± 2	149 ± 2
Potassium (mmol/L)	5.7 ± 0.5	5.3 ± 0.3	5.7 ± 0.4	5.1 ± 0.4	5.9 ± 0.4	5.1 ± 0.3
Uric acid (mg/dl)	2.7 ± 2.3	0.6 ± 0.4[c]	1.8 ± 0.5	0.4 ± 0.2[d]	1.4 ± 0.7[e]	0.4 ± 0.2[f]

[a] Values are mean ± SD. *$P < 0.05$ vs control; **$P < 0.01$ vs control.
[b] All animals in the group were <35 μmol/L.
[c] Two animals with uric acid <0.3 mg/dl were regarded as 0.3 mg/dl to calculate the mean and SD.
[d] Three animals with uric acid <0.3 mg/dl were regarded as 0.3 mg/dl to calculate the mean and SD.
[e] One animal with uric acid <0.3 mg/dl was regarded as 0.3 mg/dl to calculate the mean and SD.
[f] Four animals with uric acid <0.3 mg/dl were regarded as 0.3 mg/dl to calculate the mean and SD.

metabolized in chickens, similar to mammals such as humans, monkeys, rats, mice, and rabbits. TK parameters significantly impact toxicity: systemic exposure is often correlated with adverse effects. The values for TK parameters in the 4-wk toxicity study suggested that systemic exposure was sufficiently high to evaluate toxicity for the following three reasons: first, the mean AUC_{0-24h} and C_{max} values increased in a dose-dependent manner in the range of 12.5–62.5 mg/kg per day and there was no saturation up to 62.5 mg/kg per day; second, the AUC_{0-24h} on Day 28 at a dose of 62.5 mg/kg per day was approximately 71 times higher than the AUC_{inf} at a dose of 2.5 mg/kg in the single oral-dosing experiment and the AUC_{inf} value was equal to the AUC_{τ} at steady state; thus, the 4-wk toxicity study was conducted at doses sufficiently high to ensure an adequate evaluation of safety; and third, accumulation was observed from Days 1 to 14 with repeated administration, but no repeated dosing effects were observed from Days 14 to 28, indicating that systemic exposure did not decrease and was sufficiently maintained to ensure efficacy during repeated BXM administration for up to 28 d.

In blood chemistry analyses (Table 3), the calcium level in the 62.5 mg/kg per day dosing group was statistically lower than that of the control group on Day 27 ($P < 0.05$). However, it was judged that this change was incidental for the following reasons: the values for the 62.5 mg/kg per day dosing group on Day 27 were within the range of individual variation, and no other BXM-related changes were observed in terms of either blood chemistry or histopathology. Although a statistically lower Glu

Table 4. Mean plasma concentration, maximum plasma concentration (C_{max}), area under the plasma concentration–time curve from 0 to 24 h (AUC_{0-24h}), and time to maximum plasma concentration (T_{max}) of baloxavir acid (BXA, active form) in the 4-wk toxicity study in Julia-Lite and Julia chickens (*Gallus domesticus*, Galliformes). The sampling times were before (approximately 24 h after the previous dosing) and 2, 8, and 24 h after dosing on Days 14 and 28, and analysis was performed using samples from three or four birds at 12 and 14 wk old on Days 14 and 28, respectively. Parameters were calculated using PK plus pharmacokinetics software.

					Time after administration (h)						
					Prior to	2	8	24			
Day	Dose (mg/kg per day)	Strain	Sex	No. of birds	Plasma concentration of BXA (μg/ml)				C_{max} (μg/ml)	AUC_{0-24h} (μg · h/ml)	T_{max} (h)
14	12.5	Julia-Lite	Female	3	0.687	8.808	2.490	0.594	8.808	68.060	2.0
14	62.5	Julia-Lite	Female	4	5.861	27.412	9.962	5.824	27.412	271.650	2.0
28	12.5	Julia-Lite	Female	3	1.249	3.544	3.177	1.164	4.426	59.687	6.0
28	62.5	Julia-Lite	Female	4	15.100	11.141	14.899	9.552	17.495	299.975	4.5

value was observed on Day 27 in the 12.5 mg/kg per day group compared with the control group, this change was considered unrelated to BXM because there was no dose-dependent relationship with the 62.5 mg/kg per day group. On Day −1, although some statistically significant changes were observed in hematology and blood chemistry parameters (Tables 2 and 3), these changes were deemed unrelated to BXM because they were apparent before BXM administration. From the above-mentioned data, the results of the 4-wk toxicity study indicated that there were no BXM-related changes in any parameter evaluated and the NOAEL was estimated to be 62.5 mg/kg per day. Because of the high exposure safety margin compared with exposure at the previously reported efficacy dose 2.5 mg/kg,[42] our data also suggest that treating HPAIV-infected birds with BXM could be considered safe.

The 4-wk toxicity study was conducted to evaluate the avian-specific toxicity of BXM, the tolerability of which has already been demonstrated in mammals.[29] In previous nonclinical studies in mammals, rat-specific BXM-related changes were observed in the vitamin K–dependent coagulation system in rats and increases in plasma hepatic enzymes were observed in monkeys without any histopathologic changes.[29] Herein, however, there were no BXM-related changes in the coagulation system or signs of hepatic damage. Changes in the coagulation system in chickens were assessed by evaluating PT and hematologic and histopathologic parameters. In American kestrel (*Falco sparverius*, Falconiformes) and Japanese quail (*Coturnix japonica*, Galliformes) exposed to anticoagulant rodenticides, prolonged PT, decreased Hct, and hemorrhaging in some organs have been reported, suggesting that the parameters evaluated in this study were suitable for assessing changes in the vitamin K–dependent coagulation system.[30,32,45] Hepatic damage in chickens was assessed based on the results of blood chemistry and histopathologic analyses. Changes in AST, BA, T. Pro, Alb, Glob, and Glu were selected as hepatic damage indicators in this study because these parameters are commonly assessed in birds as measures of hepatic function.[10,12] BA levels were measured comprehensively in this study, although BA consists of many kinds of acid.[49] No changes of those parameters, as well as no histopathologic changes in the liver, suggest that no evidence of hepatotoxicity in chicken was observed.

In this study, an approximately 71 times higher exposure margin was confirmed in chickens. Regarding species differences in systemic exposure between mammals, the AUC_{0-24h} value in monkeys is at most approximately 11 times higher than that in rats with oral administration at the same dosage for 2 wk. Compared with the 11-fold differences in systemic exposure between mammals, the approximately 71 times higher exposure safety margin confirmed in chickens in the present study is high enough, taking into consideration the systemic exposure differences between avian species. The high exposure margin observed in this toxicity study suggests that other avian species used as clinical models can also be safely treated with daily oral administration of BXM for up to 2 wk, in reference to the human pharmaceutical guidelines described above. Differences in drug absorption and metabolism between chickens (Galliformes) and endangered avian species would be expected, because there are some differences in the digestive organs and metabolizing enzymes between chickens and other avian species.[20,21] Similar to differences in exposure between mammals,[29] the pharmacokinetics of BXM and BXA differ between chickens, ducks, and white-tailed eagles (*Haliaeetus albicilla*) (unreported in-house data). Pharmacokinetics data for BXA and BXM in other bird species would be helpful for determining dosage and use in those species.

To estimate the dosage for avian species from TK data, the approach used in the development of human medicines is useful. In a single-dosing study using a human influenza A virus–infected mouse model, the estimated target concentration 24 h after dosing (C_{24h}) of BXA in human plasma was >6.85 ng/ml, at which a ≥2.5-log reduction in viral titer could be expected.[2,22] In chickens, the target C_{24h} of BXA was estimated to be 28 ng/ml as the plasma concentration required for medical treatment, which was the mean C_{24h} of BXA at 2.5 mg/kg in the single-dosing TK study. Pharmacological effects would be observed when the concentration of BXA exceeds the mean C_{24h} of ≥28 ng/ml. A target C_{24h} of ≥28 ng/ml might also be standard for endangered avian species if the dosage and use of BXM were determined for other taxonomic groups (e.g., orders) of endangered avian species.

The data obtained in this study regarding the safety of BXM in avian species could aid in efforts to preserve endangered birds by using this agent. Endangered birds should be isolated during treatment for HPAI by using BXM.

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