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MAJOR ARTICLE

Emergence of Oseltamivir-Resistant H7N9 Influenza Viruses in Immunosuppressed Cynomolgus Macaques

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Antiviral compounds (eg, the neuraminidase inhibitor oseltamivir) are invaluable for the treatment of individuals infected with influenza A viruses of the H7N9 subtype (A[H7N9]), which have infected and killed hundreds of persons. However, oseltamivir treatment often leads to the emergence of resistant viruses in immunocompromised individuals. To better understand the emergence and properties of oseltamivir-resistant A(H7N9) viruses in immunosuppressed individuals, we infected immunosuppressed cynomolgus macaques with an A(H7N9) virus and treated them with oseltamivir. Disease severity and mortality were higher in immunosuppressed than in immunocompetent animals. Oseltamivir treatment at 2 different doses reduced A(H7N9) viral titers in infected animals, but even high-dose oseltamivir did not block viral replication sufficiently to suppress the emergence of resistant variants. Some resistant variants were not appreciably attenuated in cultured cells, but an oseltamivir-resistant A(H7N9) virus did not transmit among ferrets. These findings are useful for the control of A(H7N9) virus infections in clinical settings.

Keywords. Influenza virus; oseltamivir resistance; immunosuppression; nonhuman primates.

Antiviral compounds are the only specific therapeutic option to treat influenza virus infections; they are also the first line of defense against novel (pandemic) influenza viruses to which vaccine would be unavailable. Several inhibitors of the viral neuraminidase (NA) protein (including oseltamivir, zanamivir, peramivir, and laninamivir; reviewed in [1]) are available. Viruses resistant to the NA inhibitors [2, 3] are often attenuated compared with the NA inhibitor-sensitive viruses [4, 5] but can occasionally become dominant in human populations [6]. Importantly, NA inhibitor-resistant influenza viruses frequently emerge in drug-treated immunocompromised patients with delayed virus clearance [7–9].

Influenza A(H7N9) viruses emerged in 2013 and have since caused severe respiratory infections in 808 individuals and 322 deaths. Most A(H7N9) viruses tested to date are sensitive to NA inhibitors [10, 11]; however, there are reports of poor oseltamivir efficacy in an infected person [12, 13] and experimentally infected mice [14].

Immunosuppression in nonhuman primates mirrors the immune responses and pathogen susceptibility observed in immunocompromised humans [15–17], and in a recent study, oseltamivir-resistant A(H7N9) viruses were isolated from 2 of 3 influenza virus-infected nonhuman primates treated with oseltamivir [18]; however, the properties of these oseltamivir-resistant viruses were not tested and this study did not include immunocompromised animals. In the current study, we therefore assessed the emergence of oseltamivir-resistant A(H7N9) viruses in immunosuppressed, virus-infected nonhuman primates treated with oseltamivir including a high-dose regimen.

METHODS

Ethics Statement

All experiments in cynomolgus macaques were approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories (approval Nos. IACUC814-001, IACUC814-004, and IACUC814-005), and were performed in accordance with the animal welfare bylaws of Shin Nippon Biomedical Laboratories, Drug Safety Research Laboratories, which is fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care International.

Viruses

Influenza A/Anhui/1/2013 (H7N9; Anhui/1) was kindly provided by Y. Shu (World Health Organization [WHO] Collaborating Center for Reference and Research on Influenza, Chinese National Influenza Center, and National Institute for Viral Disease Control and Prevention, China Centers for Disease Control and Prevention). All experiments with H7N9 viruses were performed in enhanced biosafety level 3 containment laboratories.

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Antiviral Compounds

Animals were treated with oseltamivir phosphate (Tamiflu; Roche Laboratories). In vitro experiments were carried out with oseltamivir carboxylate, zanamivir, laninamivir, and peramivir (all kindly provided by Daiichi Sankyo).

Animals

We used approximately 2–3-year-old male cynomolgus macaques (*Macaca fascicularis*) and 6–8-month old female ferrets (Triple F farm). All animals were serologically negative for contemporary influenza A and B viruses.

Immunosuppression of Cynomolgus Macaques

Cyclophosphamide (CP; Nakalai Tesque) was intravenously administered by bolus injection. Cyclosporine A (CA; Novartis Pharma) was orally administered via catheters.

Experimental Infection and Oseltamivir Treatment of Cynomolgus Macaques

Cynomolgus macaques (*M. fascicularis*) were inoculated with Anhui/1 (10^7 plaque forming units [PFUs]/mL) through a combination of intratracheal (4.5 mL), intranasal (0.5 mL per nostril), ocular (0.1 mL per eye), and oral (1 mL) routes, resulting in a total infectious dose of 6.7×10^7 PFUs [14, 19–21].

Deep-Sequencing Analysis

Samples were prepared as described by Moncla et al [22] (see also Supplementary Data). For sequence analyses, FASTQ files were imported into CLC Genomics Workbench software, version 6 (CLC Bio) for analysis. Reads were trimmed (based on a quality scores threshold of Q30 and a minimum read length of 100 bases) and mapped to the Anhui/1 reference sequence (a minimum average coverage of 100× was required). To reduce the effects of uneven sequencing depth across samples, reads were subsampled so that all assemblies had an average coverage of approximately 1000×. Variants were called using a minimum single-nucleotide polymorphism frequency of 1%, a minimum coverage of 100 reads, and a central base quality score of Q30 or higher.

Neuraminidase Inhibition Assay

NA inhibition assays were performed as described elsewhere [23]. Briefly, viruses were mixed with NA inhibitors and incubated for 30 minutes at 37°C. Then, methylumbelliferyl-N-acetylneuraminic acid (Sigma) as a fluorescent substrate was added. One hour later, sodium hydroxide in 80% ethanol was added to stop the reaction. The fluorescence of the solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm, and the 50% inhibitory concentration (IC_{50}) was calculated using GraphPad Prism software.

Hemagglutinin Receptor-binding Specificity Assay

Viral binding was measured by biolayer interferometry on an Octet RED instrument (ForteBio). Briefly, biotinylated sialylglycopolymers were loaded onto streptavidin biosensors (ForteBio). Viruses were added at 128 hemagglutinating units per 50 µL, and viral binding to biotinylated sialylglycopolymers ("association") was measured at 30° C for 3000 seconds in the presence of 10 µmol/L oseltamivir carboxylate (Roche). Then, dissociation was measured by moving biosensors to buffer without viruses for 3000 seconds at 30°C ("dissociation").

Transmission Studies in Ferrets

Ferrets were anesthetized with ketamine and xylazine (5 and 0.5 mg/kg body weight, respectively) and infected intranasally with 10^6 PFUs of virus. On day 1 after infection, 1 naive ferret was cohoused in a perforated cage adjacent to an inoculated ferret. Nasal wash samples were collected several days after inoculation or cohousing, and viral titers were determined by means of plaque assays in Madin-Darby canine kidney cells.

RESULTS

Immunosuppression in Cynomolgus Macaques

Previously, Pham et al [17] established a protocol to suppress immune responses in cynomolgus macaques, based on a combination therapy of CP and CA, which are widely used as immunosuppressants in solid organ transplantations and cancer treatment. We note that this protocol is not intended to mimic immunosuppression in patients; it is, however, an established approach to suppress immune responses in nonhuman primates [24–26]. In the current study, we used a similar treatment regimen by treating 25 cynomolgus macaques (Table 1) with CA (50 mg/kg) daily for 7 days before virus infection, and with CP (40 mg/kg) on days 7, 5, 3, and 1 before infection (Figure 1). Before infection, we assessed body weight (Supplementary Tables S1 and S2) and body temperature (Supplementary Tables S3 and S4), and we obtained blood samples (Supplementary Figure S1). The hematological parameters of all blood samples remained above our threshold counts of 1000/µL blood for platelets, 500/µL for lymphocytes, and 250 000/µL for thrombocytes (Supplementary Figure S1).

On day 0 (ie, the day of virus infection), immunosuppressive treatment was discontinued for 5 animals (Supplementary Table S5) owing to their overall health status, although their hematological parameters were still above the threshold (Supplementary Figure S1). Starting on day 0, all other animals were treated with reduced doses of CA (Figure 1 and Supplementary Table S5). On day 1 after infection and every other day thereafter, hematological parameters were assessed, and animals were treated with 20 mg/kg of CA and CP unless ≥1 of the parameters fell below the threshold (Figure 1, Supplementary Figure S1, and Supplementary Table S5).

Based on these criteria, immunosuppression treatment was discontinued for most animals on day 0 or on day 1 after infection (Supplementary Table S5); however, we resumed CA/CP treatment if the hematological parameters improved above the

Table 1. Overview of Animal Groups, Immune Status, Virus Infection, Oseltamivir Treatment, and Outcome of Infection

Group	Immune Status[a]	Virus Infection[b]	Oseltamivir Treatment[c]	Postinfection Day		Animal ID No.
				Death	Autopsy	
1 (n = 7)	Suppressed	Yes	None	3	…[d]	4
				4	…	2
				4	…	3
				6	…	5
				…	14	1
				…	14	31
				…	14	32
2 (n = 6)	Competent	Yes	None	…	6	9
				…	6	10
				…	6	12
				…	14	7
				…	14	8
				…	14	11
3 (n = 6)	Suppressed	Yes	4 mg/kg	5	…	37
				…	6	34
				…	6	39
				7	…	38
				…	14	35
				…	14	36
4 (n = 6)	Competent	+	4 mg/kg	…	6	41
				…	6	42
				…	6	46
				…	14	40
				…	14	43
				…	14	45
5 (n = 6)	Suppressed	Yes	100 mg/kg	5	…	17
				…	6	20
				…	6	28
				…	14	16
				…	14	18
				…	14	21
6 (n = 6)	Competent	Yes	100 mg/kg	…	6	22
				…	6	23
				…	6	24
				…	14	25
				…	14	26
				…	14	27
7 (n = 3)	Suppressed	No	None	5	…	14
				…	6	13
				…	14	15
8 (n = 3)	Suppressed	No	100 mg/kg	…	6	33
				…	14	29
				…	14	30

Abbreviation: ID, identification.

[a]Nonhuman primates were treated with cyclosporine A and cyclophosphamide, as described in the text and shown in Figure 1.

[b]Animals were infected with 6.7×10^7 plaque-forming units of Anhui/1 virus, as described in the text.

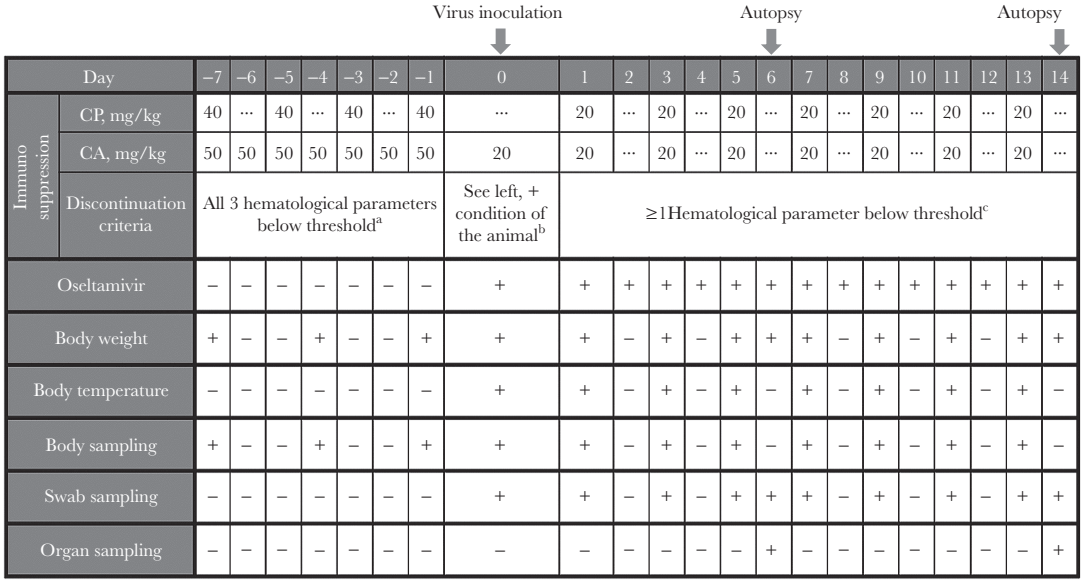
[c]Macaques were treated with the indicated doses of oseltamivir. as described in the text.

[d]Not applicable.

threshold levels. Leukocyte and thrombocyte counts began to rebound by day 7 after infection in some animals; however, the lymphocyte counts of all immunosuppressed animals remained low during the infection period (Supplementary Figure S1). These findings demonstrate robust immunosuppression in cynomolgus macaques upon combination therapy with CA and CP. For controls, 18 age-matched animals were treated with phosphate-buffered saline (Table 1).

A(H7N9) Virus Infection in Immunosuppressed Macaques

First, we compared A(H7N9) pathogenicity between immunocompetent and immunosuppressed animals. Macaques in



Day		−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Immunosuppression	CP, mg/kg	40	···	40	···	40	···	40	···	20	···	20	···	20	···	20	···	20	···	20	···	20	···
	CA, mg/kg	50	50	50	50	50	50	50	20	20	···	20	···	20	···	20	···	20	···	20	···	20	···
	Discontinuation criteria	All 3 hematological parameters below threshold[a]							See left, + condition of the animal[b]	≥1Hematological parameter below threshold[c]													
Oseltamivir		−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Body weight		+	−	−	+	−	−	+	+	+	−	+	−	+	+	+	−	+	−	+	−	+	+
Body temperature		−	−	−	−	−	−	−	+	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Body sampling		+	−	−	+	−	−	+	+	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Swab sampling		−	−	−	−	−	−	−	+	+	−	+	−	+	+	+	−	+	−	+	−	+	+
Organ sampling		−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+

[a]Blood samples were tested on days −7, −4, and −1. The thresholds were set at 1000/mL for leukocyte, 500/mL for lymphocyte and 250 000/mL for platelet count; if all 3 hematological parameters fell below the threshold, immunosuppression treatment was discontinued; [b]In addition to the hematological parameters (thresholds), the health of the animal was evaluated; [c]Blood samples were tested on day 1 and every other day thereafter. Immunosuppression treatment was discontinued if 1 hematological parameter fell below the threshold.

Figure 1. Overview of study design. Macaques were treated with cyclophosphamide (CP) and cyclosporine A (CA), as indicated. Oseltamivir treatment was initiated immediately before infection with Anhui/1 virus, as described in the text. On the indicated days, nasal, tracheal, oral, rectal, and conjunctival swab samples were collected.

groups 1 and 2 (Table 1) were infected with 6.7×10^7 PFUs of the prototype A(H7N9) virus A/Anhui/1/2013 (Anhui/1) [27, 28]. On days 6 and 14 after infection, 3 animals in each group were euthanized for viral titration in various organs, unless they had previously succumbed to the virus infection (Table 1); before euthanasia, clinical conditions were assessed, and blood and swab samples were taken. Hematological parameters were monitored as described above.

Survival after Anhui/1 virus infection correlated with the immune status of the infected animals. All immunocompetent animals survived infection (group 2; Tables 1 and 2), whereas 4 of 7 immunosuppressed animals died of their infection (group 1; Tables 1 and 2). Body temperatures differed between groups 1 and 2 on day 0, but not on other days (Supplementary Table S4*C*). Body weight loss was significantly higher in immunosuppressed animals (group 1) than in immunocompetent animals (group 2) several days before virus infection (Supplementary Table S2*A*), suggesting that immunosuppression, rather than virus infection, caused this difference. The viral titers in nasal (but not in tracheal) swab samples were significantly higher in group 1 than in group 2 on day 9 after infection, but not at earlier time points after infection (Table 2; Supplementary Table S6*A*). Lung viral titers on day 6 after infection seemed slightly higher in immunocompromised than in immunocompetent animals, although this difference was not statistically significant (Table 2 and Supplementary Table S7). Thus, Anhui/1 virus infection is more severe in immunosuppressed macaques than in immunocompetent animals, consistent with observations in immunocompromised humans infected with influenza viruses [29].

Effect of Oseltamivir on Influenza Severity in Immunocompromised Macaques

To test the benefits of oseltamivir treatment in immunosuppressed macaques, immunocompetent and –suppressed animals (Table 1) were infected with Anhui/1 virus. Immediately before virus inoculation, and every day thereafter, animals were also treated with a low (4 mg/kg; groups 3 and 4) or high (100 mg/kg; groups 5 and 6) dose of oseltamivir; the lower dose is comparable to the recommended therapeutic dose in humans.

Oseltamivir treatment of immunosuppressed macaques slightly improved the outcome of infection; however, these differences were not statistically significant. Oseltamivir treatment did not have significant effects on body weight or temperature (Supplementary Tables S1, S2*D,* S2*E,* S3, S4*A,* and S4*B*), with the exception of a low body temperature in group 2 animals on day 0. However, for immunocompetent and immunosuppressed animals, treatment with oseltamivir at 4 and/or 100 mg/kg resulted in significantly lower viral titers than non–oseltamivir-treated animals in nasal and/or tracheal swab samples on days 1, 3, 5, and/or 7 after infection (Table 2; Supplementary Table S8). Oseltamivir treatment also reduced viral titers in the bronchus and trachea but did not significantly reduce day 6 lung viral titers (Table 2 and Supplementary Table S9).

Table 2. Viral Titers in Respiratory Swab and Tissue Samples of Immunocompetent or Immunosuppressed Macaques Infected With Anhui/1 Virus and Treated With Oseltamivir as Indicated[a]

	Viral Titers of Animals Infected With Anhui/1 Virus, Log_{10} PFUs/mL (Swab Samples) or Log_{10} PFUs/g (Tissue Samples)																																		
Sample Type by Postinfection Day	Group 1 (Immunosuppressed; No Oseltamivir)							Group 2 (Immunocompetent; No Oseltamivir)						Group 3 (Immunosuppressed; Oseltamivir, 4 mg/kg)						Group 4 (Immunocompetent; Oseltamivir, 4 mg/kg)						Group 5 (Immunosuppressed; Oseltamivir, 100 mg/kg)						Group 6 (Immunocompetent; Oseltamivir, 100 mg/kg)					
Animal ID No.	4	2	3	5	1	31	32	9	10	12	7	8	11	37	34	39	38	35	36	41	42	46	40	43	45	17	20	28	16	18	21	22	23	24	25	26	27
Day of death or autopsy[b]	3 (D)	4 (D)	4 (D)	6 (D)	14 (A)	14 (A)	14 (A)	6 (A)	6 (A)	6 (A)	14 (A)	14 (A)	14 (A)	5 (D)	6 (A)	6 (A)	7 (D)	14 (A)	14 (A)	6 (A)	6 (A)	6 (A)	14 (A)	14 (A)	14 (A)	5 (D)	6 (A)	6 (A)	14 (A)	14 (A)	14 (A)	6 (A)	6 (A)	6 (A)	14 (A)	14 (A)	14 (A)
Swab samples[c]																																					
Day 1: nasal	6.3	5.6	6.5	5.6	4.6	4.2	4.2	4.4	4.2	5.0	5.3	4.6	3.7	2.3	4.7	3.1	3.5	4.2	4.5	2.3	3.6	1.6	3.7	3.4	2.4	1.0	2.7	2.5	…	2.4	3.3	4.4	2.4	3.3	2.7	1.0	4.7
Day 1: trachea	4.1	3.0	6.0	2.8	2.8	3.5	2.7	3.8	…	2.2	3.5	3.8	1.0	2.1	1.9	1.3	1.5	2.6	1.7	…	2.4	1.6	3.5	…	2.2	…	…	…	…	1.8	…	…	…	…	…	…	…
Day 3: nasal	4.8	1.8	4.3	4.3	2.4	…	2.1	1.6	1.8	1.3	3.8	2.0	4.3	…	2.4	1.0	1.6	1.8	3.7	…	1.7	1.0	1.8	1.5	1.0	…	1.0	…	…	…	…	…	1.3	1.5	4.0	1.3	…
Day 3: trachea	3.0	2.4	3.3	…[d]	…	…	…	1.0	…	…	1.8	1.3	…	…	2.4	…	…	…	1.0	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Day 4: nasal		4.3	4.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Day 4: trachea		1.8	4.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Day 5: nasal				5.1	5.6	…	3.4	3.2	3.0	4.5	5.6	6.4	4.5	…	…	1.5	1.8	…	2.5	…	2.0	…	…	…	…	…	1.5	…	…	…	…	…	…	…	…	…	…
Day 5: trachea				1.6	3.5	…	…	…	…	1.0	2.2	2.1	3.9	…	…	1.0	…	…	1.7	…	…	…	…	…	1.0	1.0	…	…	…	…	…	…	…	…	…	…	…
Day 6: nasal				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		…	3.4	ND	ND	ND	1.0	3.7	…	ND	ND	ND		3.6	…	ND	ND	ND	…	2.5	…	ND	ND	ND
Day 6: trachea				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		…	1.9	ND	ND	ND	…	…	…	ND	ND	ND		…	…	ND	ND	ND	…	…	…	ND	ND	ND
Day 7: nasal					2.2	3.5	3.6				3.0	2.6	3.4				3.5	1.0	3.4				1.0	…	1.9				…	…	…				…	…	…
Day 7: trachea					4.0	1.7	…				2.2	2.3	3.3				…	…	…				…	…	…				…	…	…				…	…	…
Day 9: nasal					1.5	2.1	…				…	…	…					…	2.3				…	…	…				…	…	…				…	…	…
Day 9: trachea					…	…	1.6				…	…	…					…	1.3				…	…	…				…	…	…				…	…	…
Day 11: nasal					…	…	…				…	…	…					…	3.2				…	1.0	…				…	…	…				…	…	…
Day 11: trachea					…	…	…				…	…	…					…	1.0				…	…	…				…	…	…				…	…	…
Tissue samples																																					
NT	7.2	6.7	7.1	7.1	…	…	…	6.4	7.3	5.1	…	…	…	2.5	3.3	2.9	5.3	…	5.3	…	5.8	…	…	…	…	1.9	6.7	…	…	…	…	…	5.6	…	…	…	…
Trachea	6.3	5.9	5.8	6.6	…	…	…	4.5	3.2	…	…	…	…	4.0	4.0	3.4	…	…	…	…	2.5	…	…	…	…	2.6	2.4	…	…	…	…	…	…	…	…	…	…
Bronchus																																					
Right	6.0	5.6	6.0	3.6	…	…	…	4.6	3.8	2.5	…	…	…	3.5	3.4	3.4	…	…	…	…	2.4	…	…	…	…	3.4	2.6	…	…	…	…	…	…	…	…	…	…
Left	6.8	5.9	5.8	6.7	…	…	…	5.5	5.5	3.4	…	…	…	3.0	3.7	3.4	…	…	…	…	…	…	…	…	…	2.4	3.2	…	…	…	…	…	…	…	…	…	…
Lung																																					
RU	7.2	6.5	4.5	4.3	…	…	…	…	3.6	…	…	…	…	5.4	5.1	6.7	5.1	…	…	3.2	…	…	…	…	…	6.1	…	6.7	…	…	…	…	…	…	…	…	…
RM	7.2	5.7	5.2	4.6	…	…	…	…	2.6	…	…	…	…	3.7	4.3	6.0	5.3	…	…	3.5	…	2.8	…	…	…	…	…	6.5	…	…	…	…	…	2.4	…	…	…
RL	6.1	6.6	5.7	3.5	…	…	…	3.1	5.7	…	…	…	…	3.9	6.6	6.5	5.3	…	…	…	5.3	…	…	…	…	6.3	…	6.7	…	…	…	…	…	…	…	…	…
LU	7.4	5.3	3.1	3.2	…	…	…	…	…	…	…	…	…	3.7	7.3	2.6	…	…	…	2.4	…	…	…	…	…	5.2	3.5	…	…	…	…	2.9	3.8	…	…	…	…
LM	3.2	2.7	2.9	3.4	…	…	…	2.9	…	…	…	…	…	3.7	6.5	…	…	…	…	2.8	…	…	…	…	…	6.3	3.2	…	…	…	…	…	2.9	…	…	…	…
LL	5.1	6.2	3.1	2.3	…	…	…	4.6	…	…	…	…	…	3.3	6.7	6.6	2.3	…	…	3.7	…	…	…	…	…	…	7.8	…	…	…	…	2.8	3.3	…	…	…	…
Brain																																					
Frontal	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Parietal	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Temporal	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Occipital	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Cerebellum	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	2.0	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Brain stem	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1.8	…	…	…	2.9	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Olfactory bulb	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

Table 2. Continued

	Viral Titers of Animals Infected With Anhui/1 Virus, Log_{10} PFUs/mL (Swab Samples) or Log_{10} PFUs/g (Tissue Samples)																													
Sample Type by Postinfection Day	Group 1 (Immunosuppressed; No Oseltamivir)							Group 2 (Immunocompetent; No Oseltamivir)							Group 3 (Immunosuppressed; Oseltamivir, 4 mg/kg)							Group 4 (Immunocompetent; Oseltamivir, 4 mg/kg)							Group 5 (Immunosuppressed; Oseltamivir, 100 mg/kg)							Group 6 (Immunocompetent; Oseltamivir, 100 mg/kg)						
Animal ID No.	4	2	3	5	1	31	32	9	10	12	7	8	11	37	34	39	38	35	36	41	42	46	40	43	45	17	20	28	16	18	21	22	23	24	25	26	27
Day of death or autopsy[b]	3 (D)	4 (D)	4 (D)	6 (D)	14 (A)	14 (A)	14 (A)6 (A)	6 (A)	6 (A)	6 (A)	14 (A)	14 (A)	14 (A)5 (D)		6 (A)	6 (A)	7 (D)	14 (A)	14 (A)6 (A)		6 (A)	6 (A)	14 (A)	14 (A)	14 (A)5 (D)		6 (A)	6 (A)	14 (A)	14 (A)	14 (A)6 (A)		6 (A)	6 (A)	14 (A)	14 (A)	14 (A)	
Tonsil	3.3	3.0	4.4	5.3	4.9	…	…	4.5	4.3	2.5	…	…	…	…	…	2.9	…	4.0	…	…	5.2	3.3	…	…	…	…	3.4	…	…	…	…	…	5.5	…	…	…
Mediastinal LNs	…	4.0	2.8	2.3	…	…	…	…	2.4	…	…	…	…	…	…	…	…	…	…	…	1.7	…	…	…	…	…	3.7	1.3	…	…	…	…	0.8	…	…	…	…
Heart	2.5	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	3.1	…	…	…	2.5	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Spleen	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Kidney	…	…	…	3.2	…	…	…	2.5	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Liver	1.7	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Duodenum	…	2.4	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
Rectum	…	2.2	…	2.2	…	…	…	…	2.2	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…

Abbreviations: A, autopsy; D, death; LL, left lower; LM, left middle; LNs, lymph nodes; LU, left upper; ND, not determined; NT, nasal turbinate; PFUs, plaque-forming units; RL, right lower; RM, right middle; RU, right upper.

[a]Cynomolgus macaques were inoculated with 6.7×10^7 PFUs (6.7 mL) of Anhui/1 virus through multiple routes. Up to 3 macaques per group (depending on the number of surviving animals) were euthanized on day 6 or 14 after infection. Viral titers were determined by means of plaque assays; the detection limits were 1 log_{10} PFUs/mL and 0.8 log_{10} PFUs/g for swab and tissue samples, respectively.

[b]Day of death (D) or autopsy (A) represents postinfection day.

[c]Swab samples collected on days 13 and 14 were negative for Anhui/1 virus and are not shown.

[d]Throughout table, ellipses (…) indicate that virus was not detected; blank cells, not applicable.

Histopathological analysis of selected lung samples showed no major differences in lung inflammation scores, but more virus antigen-positive cells were detected in immunosuppressed animals, consistent with higher viral titers in these animals (Supplementary Figure S2).

Emergence of Mutations That May Confer Resistance to Oseltamivir

Oseltamivir blocks the sialidase activity of the viral NA protein, which is essential for efficient virus release from host cells. Resistance to oseltamivir is typically caused by amino acid changes in the NA catalytic center and/or the structural framework that stabilizes the enzymatic pocket of NA.

To assess the emergence of resistant variants in oseltamivir-treated macaques, we Sanger sequenced the NA genes of influenza viruses isolated from the respiratory organs of oseltamivir-treated animals; for comparison, we also Sanger and deep sequenced the NA gene of the virus stock. With the exception of the R294K mutation (N9 numbering) in animal 36, all mutations listed in Table 3 were detected as mixed sequences with the respective wild-type nucleotides. R294K and R226K confer oseltamivir resistance to NA of the N1, N2, and N9, and N2 subtypes, respectively (reviewed in [30]). The I224T mutation affects the NA structural framework (Figure 2) and confers oseltamivir resistance to influenza B viruses [31]. These mutations were found in immunosuppressed but not immunocompetent animals (Table 3), consistent with the observation that oseltamivir-resistant viruses emerge more frequently in the former group [7]. However, treatment with a high dose of oseltamivir did not prevent the emergence of oseltamivir-resistance mutations. A T10I mutation (detected in several samples) was present in the virus stock and is therefore not listed in Table 3.

To gain additional insight into the emergence of oseltamivir resistance, we performed deep-sequencing analysis of nasal and tracheal swab samples from animals 20 and 36, which contained viruses with the R226K and/or R294K mutations. We were unable to obtain deep-sequencing data from lung samples. With the exception of the R226K and R294K changes, most mutations detected at a frequency of ≥1% fluctuated in frequency and remained at low frequencies during infection (Supplementary Table S10 and Supplementary Figure S3), suggesting that they likely do not provide a strong selective advantage in our experimental setting.

The R226K and R294K mutations were not detected at ≥1% in the virus stock, suggesting their emergence in animals 20 and 36. In animal 36, the R294K mutation was first detected in a nasal swab sample obtained on day 5; it was also predominant in nasal and tracheal swab samples from day 7 onward and in nasal turbinates on day 14 (Supplementary Table S10).

The K465R mutation was detected in the virus stock and in several samples derived from several infected macaques (Table 3 and Supplementary Table S10). However, it did not increase in frequency during the course of infection in macaques and is

Table 3. Neuraminidase Mutations Detected in Samples Obtained From Oseltamivir-Treated Macaques

Animal ID No.	Immune Status	Oseltamivir Treatment, mg/kg	Sample Type	Postinfection Day	NA Mutation(s)
16	Suppressed	100	Lung (right lower)[a]	14	G40S/**R294K**[b]
			Lung (left lower)[c]	14	G40S, **R294K**
			Lung (left middle)[c]	14	G40S, **R294K**
20	Suppressed	100	Nasal swab	5	**R226K**
			Nasal turbinate[d]	6	**R226K**, **R294K**
			Bronchus[e]	6	**R294K**, K437E
22	Competent	100	Nasal swab	5	K465R
25	Competent	100	Nasal swab	3	K465R
36	Suppressed	4	Nasal swab	11	**R294K**
			Nasal swab isolate (MDCK)[f]	13, 14	
			Nasal turbinate isolate (MDCK)[f]	14	
			Lung (right middle)	14	I305V
37	Suppressed	4	Tracheal swab	5	A370T
			Lung (left middle)	5	I305V
			Lung (left lower)	5	I305V
39	Suppressed	4	Nasal swab[g]	6	**I224T**, K465R
			Lung (left upper)	6	S404C
			Lung (left middle)[h]	6	S161D, K437E
40	Competent	4	Lung (left lower)	14	G40E
41	Competent	4	Lung (left middle)	6	S404C
42	Competent	4	Nasal swab	6	K465R
			Lung (right upper)[i]	6	T51I, K465R
46	Competent	4	Lung (left upper)	6	T65I

Abbreviations: ID, identification; MDCK, Madin-Darby canine kidney; NA, neuraminidase.

[a]Based on molecular cloning and Sanger sequencing, the R294K/G40S mutations were detected in the same NA complementary DNA (cDNA) clone.

[b]Mutations in influenza A or B virus NA proteins that confer resistance to oseltamivir are shown in boldface type.

[c]Molecular cloning of NA cDNAs was not carried out.

[d]Based on molecular cloning and Sanger sequencing, the R224K and R294K mutations were detected in different NA cDNA clones.

[e]The R294K mutation was detected by Sanger sequencing but not in the clonal analysis of 5 individual NA cDNA clones.

[f]Virus was not detected by plaque assay (below detection limit) but was detected after amplification in MDCK cells.

[g]The K465R mutation was detected by Sanger sequencing but not in the clonal analysis of 10 individual NA cDNA clones.

[h]The S161D mutation was detected by Sanger sequencing but not in the clonal analysis of 7 individual NA cDNA clones.

[i]The K465R mutation was detected by Sanger sequencing but not in the clonal analysis of 6 individual NA cDNA clones.

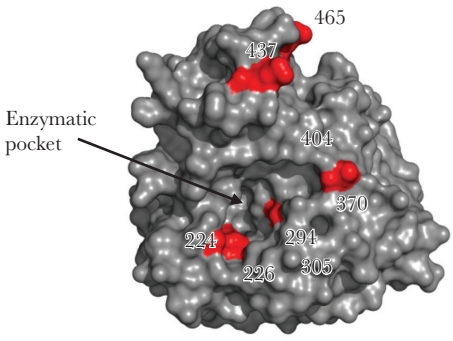


Figure 2. Location of neuraminidase (NA) mutations detected in oseltamivir-resistant viruses in the 3-dimensional structure of NA. Shown is the 3-dimensional structure of the NA monomer of A/Anhui/1/2013 virus (Protein Data Bank accession No. 4MWJ). Surface amino acids affected by the detected mutations are shown in red with black numbers, and the approximate location of mutations not located on the surface is indicated by white numbers.

therefore unlikely to be important for resistance to oseltamivir. Deep sequencing also detected the T10I mutation in several samples, which was also detected in the virus stock used and not included in Supplementary Table S10.

Susceptibility of Mutant A(H7N9) Viruses to Neuraminidase Inhibitors

We [14] and others [12] demonstrated that the R294K mutation renders A(H7N9) viruses resistant to oseltamivir. Anhui/1 encoding the NA-R226K mutation had very low NA activity (Table 4) and could not be tested for its sensitivity to NA inhibitors. For all of the other mutants, we measured sensitivity to oseltamivir by using an enzyme-based NA inhibition assay [23]. As expected, the R294K mutant was highly resistant to oseltamivir (Table 4). The additional NA-G40S mutation (found in animal 16) may have contributed to the viral resistance to oseltamivir. The I224T mutation (which renders

Table 4. Susceptibility to Neuraminidase (NA) Inhibitors of NA Mutants Detected in Oseltamivir-Treated Cynomolgus Macaques

	IC_{50}, mean (SD), nmol/L[a]				
NA Mutation	Oseltamivir Carboxylate	Zanamivir	Laninamivir	Peramivir	Location of NA Mutation
Wild type	2.1 (1.1)	3.8 (1.1)	3.0 (2.6)	0.3 (0.3)	…
G40S	1.6 (1.6)	ND	ND	ND	Stalk
T51I	1.8 (1.0)	ND	ND	ND	Stalk
T65I	2.0 (0.6)	ND	ND	ND	Stalk
S161D	1.4 (0.9)	3.8 (0.9)	4.0 (2.4)	0.2 (0.2)	Head
I224T	11.3 (6.9)	10.3 (1.0)	4.2 (4.7)	0.4 (0.4)	NA structural framework
R226K	N/A[b]	N/A	N/A	N/A	NA enzymatic pocket
R294K	10400 (3700)	161 (33	66.3 (55)	232 (54)	NA enzymatic pocket
G40S/R294K[c]	7900 (3000)	162 (35	43.2 (2.7)	271 (180)	NA enzymatic pocket(R294K)
I305V	1.2 (0.7)	2.7 (0.1	3.8 (1.6)	0.3 (0.1)	Head
A370T	0.5 (0.6)	4.2 (0.3	3.0 (1.5)	0.3 (0.0)	Head
S404C	2.3 (1.1)	13.9 (3.0	2.9 (2.0)	0.15 (0.1)	Head
K437E	1.6 (1.3)	2.4 (0.6	2.0 (1.7)	0.07 (0.0)	Head
K465R	1.9 (1.8)	4.7 (1.9	2.9 (2.0)	0.4 (0.2)	Head

Abbreviations: IC_{50} 50% inhibitory concentration; N/A, not applicable; NA, neuraminidase; ND, not determined; SD, standard deviation.

[a]Mean value of duplicate reactions. Oseltamivir carboxylate is the active form of oseltamivir, and laninamivir is the active form of laninamivir octanoate.

[b]NA activity was too low to detect in the assay.

[c]The G40S and R294K mutations were found together in viruses obtained from lung samples of animal 16.

influenza B viruses resistant to oseltamivir [31]) resulted in a 5-fold increase in the IC_{50} value compared with the wild-type virus. (based on WHO guidelines, however, reduced inhibition is defined as a 10–100-fold increase in IC_{50} for influenza A viruses). The remaining NA mutants tested did not confer resistance to oseltamivir.

In addition to oseltamivir (the most widely used NA inhibitor), we also tested the NA inhibitors zanamivir, laninamivir, and peramivir. Peramivir is structurally similar to oseltamivir but is administered intravenously. Zanamivir differs in its structure from oseltamivir; resistance to it is very rare but has been reported [32]. Resistance to laninamivir (a long-lasting NA inhibitor) is also very rare [33–35].

The R294K mutation confers moderate resistance to zanamivir, consistent with the findings of Hai et al [36]. Importantly, the R294K mutation also conferred resistance to peramivir and moderate resistance to laninamivir, as described by Meijer et al [37]. The levels of resistance to these 3 compounds may be further increased by the additional G40S mutant. These findings indicate that the R294K mutation in an N9 NA confers (at least moderate) resistance to all major NA inhibitors. The emergence of such variants in immunocompromised patients would thus be a considerable threat to public health.

Mutations in the Viral Hemagglutinin Protein

The sialic acid–cleaving function of NA must be balanced with the sialic acid–binding function of the viral hemagglutinin (HA) protein, which is critical for viral binding to host cell receptors. We therefore used Sanger sequencing of individual clones to assess the sequences of the HA genes of viruses derived from animals 16, 20, 36, and 39, whose NA genes encode oseltamivir resistance-conferring mutations (Supplementary Table S11 and Supplementary Figure S4).

The N123D (numbers refer to the amino acid position in H7 after the removal of the N-terminal signal peptide) and N149D mutations both localize to the rim of the receptor-binding pocket and affect viral receptor-binding properties [38]. The A125T mutation also localizes to the rim of the receptor-binding pocket. Based on clonal analyses of HA complementary DNA products, all 3 mutations existed in the inoculum [14], indicating that these mutations did not emerge during viral replication in cynomolgus macaques to balance the effects of oseltamivir-resistance mutations. The G133E mutation also localizes to the rim of the HA receptor-binding site and affects influenza viral receptor binding [39]; it was not detected in the virus stock, suggesting that it arose during viral replication in the animal. The G196E mutation is not located in the vicinity of the receptor-binding pocket; nonetheless, we compared viruses expressing wild-type or mutant HA-G196E for their binding ability to α2,3- or α2,6-linked sialic acids (ie, avian- or human-type receptors). No significant differences were detected between the viruses (Supplementary Figure S5). Control avian A/duck/Alberta/35/76 (H1N1) and human A/Kawasaki/173/2001 (H1N1) viruses bound to α2,3- and α2,6-linked sialic acids, respectively, as expected (Supplementary Figure S5).

Replication Kinetics of Oseltamivir-Resistant Anhui/1 Viruses in Mammalian Cells

Many, but not all, oseltamivir-resistant viruses are attenuated in vitro and/or in vivo [4]. We therefore tested the replicative potential of oseltamivir-resistant viruses in mammalian cells.

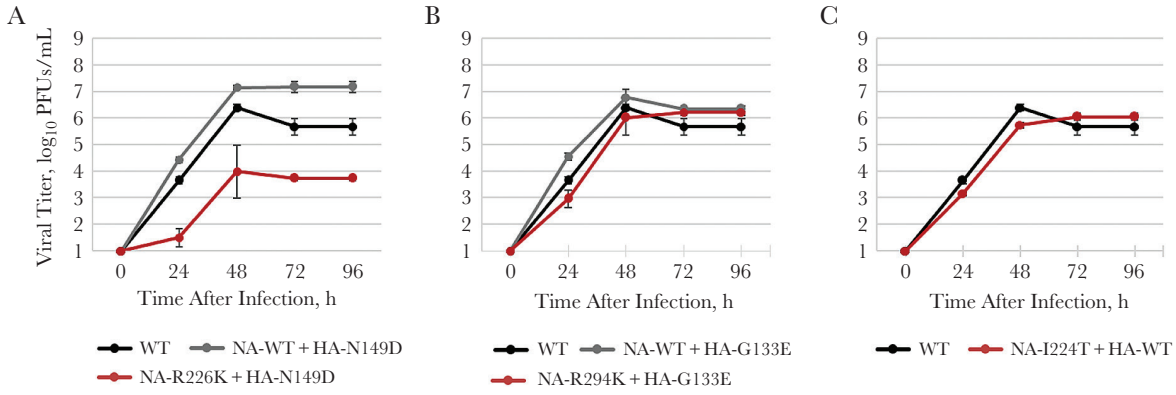


Figure 3. Viral titers of wild-type (WT) and mutant viruses in A549 cells. Cells were infected at a multiplicity of infection of 0.0001 with the indicated viruses; for example, NA-R226K + HA-N149D denotes an Anhui/1 virus expressing the NA-R226K and HA-N149D mutations. Viral titers in the cell culture supernatants were determined at the indicated time points by use of plaque assays in Madin-Darby canine kidney cells. Abbreviations: HA, hemagglutinin; NA, neuraminidase; PFUs, plaque-forming units.

We generated virus NA-R294K + HA-G133E, which possesses the mutations found in a nasal swab samples from animal 36; virus NA-WT + HA-G133E (possessing wild-type NA) served as a control. The NA-WT + HA-N149D and NA-R226K + HA-N149D viruses were generated to test mutations detected in animal 20. In animal 39, the NA-I224T mutation was found with wild-type HA or HA encoding N123D and/or N149D. Because the virus population mainly encoded wild-type HA, we generated an Anhui/1 virus encoding NA-I224T in combination with wild-type HA (termed NA-I224T + HA-WT).

In adenocarcinomic human alveolar basal epithelial (A549) and Madin-Darby canine kidney cells, NA-R226K + HA-N149D virus had significantly impaired replicative ability compared with NA-WT + HA-N149D virus (Figure 3A and Supplementary Figure S6*A*), presumably because of the low NA activity conferred by the NA-R226K mutation. This mutant is therefore unlikely to spread in nature. The NA-R294K + HA-G133E virus replicated to slightly lower titers than NA-WT + HA-G133E virus (Figure 3B and Supplementary Figure S6*B*) but still replicated as well as the wild-type virus. The growth properties of the NA-I224T + HA-WT virus did not differ substantially from those of the wild-type virus (Figure 3C and Supplementary Figure S6*C*). Thus, with the exception of the NA-R226K mutant, the viruses possessing NA inhibitor resistance-conferring mutations replicated as well as the wild-type virus.

Transmissibility of Oseltamivir-Resistant Anhui/1 Virus in Ferrets

To assess the transmissibility of oseltamivir-resistant Anhui/1 virus, we inoculated ferrets with 10^6 PFUs of the NA-R294K + HA-G133E virus, which is resistant to oseltamivir and encodes an HA mutation that increases H5N1 viral binding to human receptors [40]. One day later, we cohoused naive ferrets in cages with a perforated divider next to inoculated ferrets; under these conditions, wild-type Anhui/1 virus replicated well in the nasal turbinates of ferrets and transmitted via respiratory droplets in 1 of 3 pairs of ferrets [14]. Nasal wash samples were collected from the inoculated and exposed ferrets several days after infection or cohousing, respectively, to assess viral titers (Supplementary Table S12). We detected robust viral replication in all 3 infected animals until 5 days after inoculation. By contrast, exposed animals did not seroconvert and no virus was isolated from them, indicating that the oseltamivir-resistant Anhui/1 virus does not readily spread among ferrets by respiratory droplets.

DISCUSSION

In the current study, we assessed the benefits of oseltamivir treatment and the emergence of oseltamivir-resistant variants in an immunosuppressed nonhuman primate model. The immunosuppression protocol we used is established for nonhuman primates [17] and causes weight loss and decreased hematological parameters. Oseltamivir treatment improved survival and reduced the severity of disease in immunocompromised macaques, but it also resulted in the emergence of oseltamivir-resistant variants, as has been reported for immunocompromised individuals infected with seasonal influenza viruses [41] and immunosuppressed ferrets [42].

The resistant variants were not attenuated in their replicative ability in cultured cells, with the exception of 1 mutant. However, a virus with a mutation in HA and a mutation in NA that confers resistance to NA inhibitors did not transmit among ferrets via respiratory droplets. It remains unknown whether the lack of respiratory droplet transmission of this mutant is due to the fact that humans, ferrets, and nonhuman primates differ in the distribution and prevalence of sialyloligosaccharides in their respiratory tract. Alternatively, this mutant may be attenuated in its replicative ability in ferrets, even though its replication kinetics were similar to those of wild-type virus in cultured cells (Figure 3 and Supplementary Figure S6). Collectively, our data highlight the benefits and risks of oseltamivir treatment and establish immunosuppressed cynomolgus macaques as a

robust model for the study of the emergence of drug resistance in influenza virus-infected mammals.

Two previous studies compared the benefits of drug treatment side by side in immunocompromised and immunocompetent mice [43, 44]. Both studies detected benefits of drug treatment; however, drug treatment did not result in virus clearance in immunocompromised animals. In 1 study, drug-resistant influenza viruses were isolated from drug-treated immunocompetent and -compromised animals [43]; the other study did not assess the emergence of drug-resistant variants [44]. The data presented and discussed here and those obtained from patients [45–48] differ in the antiviral compounds, treatment strategies, and influenza virus strains. Still, they demonstrate the benefits of antiviral compounds in immunocompromised hosts while highlighting the emergence of drug-resistant variants.

Supplementary Data

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

Notes

Author contributions. M. K., K. I. H., S. Y., R. U., M. I., N. N., and Y. T. performed the experiments. E. K. and T .J .S .L. performed the statistical analyses. L. H. M. and T. C. F. performed deep sequencing and deep-sequencing data analysis. M. K., S. Y., N. N., M. I., S. F., Y. I., K. O, T. W., T. C. F., G. N., and Y. K. designed the experiments, analyzed the data, and wrote the manuscript.

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Potential conflicts of interest. G. N. is a cofounder of FluGen and has received royalties from MedImmune. She holds the following patents: Recombinant influenza viruses for vaccines and gene therapy (US patent 8,715,940 B2); Filovirus vectors and noninfectious filovirus-based particles (US patent 7,211,378); and Recombinant influenza vectors with tandem transcription units (US patent 7968101B2). Y. K. is a cofounder of FluGen and has received grant support from Chugai Pharmaceuticals, Daiichi Sankyo Pharmaceutical, Toyama Chemical, Tauns Laboratories, Denka Seiken, and Ttsumura and royalties from MedImmune. He holds the following patents: Recombinant influenza viruses for vaccines and gene therapy (US patent 8,715,940 B2); Viruses comprising mutant ion channel protein (US patent 6,872,395); Mutant cells with altered sialic acid (US patent 7,176,021); Filovirus vectors and noninfectious filovirus-based particles (US patent 7,211,378); Signal for packaging of influenza virus vectors (US patent 7585657B2); Viruses encoding mutant membrane protein (US patent 7588769B2); Recombinant influenza vectors with a polII promoter and ribozymes for vaccines and gene therapy (US patent 7723094); Recombinant influenza vectors with tandem transcription units (US patent 7968101B2); Adenoviral vectors for influenza virus production (US patent 8,043,856); Viruses comprising mutant ion channel protein (US patent 8,057,806); Cell-based systems for producing influenza vaccines (US patent 8,163,523); Influenza B viruses with reduced sensitivity to neuraminidase (NA) inhibitor (US patent 8,465,960); High titer recombinant influenza viruses for vaccine and gene therapy (US patent 8,475,806 B2); Influenza A virus with attenuating mutations in NS2 protein (US patent 8,507,247); and NA-deficient live influenza vaccines (US patent 8,597,661). All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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