

Efficacy of Neuraminidase Inhibitors against H5N6 Highly Pathogenic Avian Influenza Virus in a Nonhuman Primate Model.

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journal or publication title	Antimicrobial agents and chemotherapy
volume	64
number	7
year	2020-06-23
URL	http://hdl.handle.net/10422/00012751

doi: 10.1128/AAC.02561-19(<https://doi.org/10.1128/aac.02561-19>)

Table S1. Schedule of the present study.

	Days after virus inoculation							
	0	1	2	3	4	5	6	7
Swab sampling	✓	✓	✓	✓	✓	✓	✓	✓
Blood sampling	✓	✓		✓		✓		✓
Antivirals/saline administration		✓	✓	✓	✓	✓		

Cynomolgus macaques were challenged with H5N6 HPAIV on day 0. Swab and/or blood samplings were done before virus infection and before antiviral drugs/saline administration on indicated days. Cynomolgus macaques were autopsied on day 7.

Table S2. Virus titers in tissue samples from cynomolgus macaques on day 7 after A/black swan/Akita/1/2016 (H5N6) virus infection

Tissue	Virus titer (\log_{10} TCID ₅₀ /g) on day 7 post infection											
	Saline			Oseltamivir			Peramivir			Amantadine		
	S1 ^a	S2	S3	O1	O2	O3	P1	P2	P3	A1	A2	A3
Nasal mucosa	2.50	2.67	< ^b	<	<	<	<	<	<	<	3.50	<
Oronasopharynx	3.50	4.00	≤ 2.00 ^e	<	<	<	≤ 1.67 ^c	2.50	<	<	≤ 1.83 ^d	<
Right tonsil	2.67	3.67	4.00	<	<	<	4.50	4.67	<	<	<	<
Left tonsil	4.56	3.77	3.83	<	<	<	≤ 2.00	4.77	≤ 2.00	<	≤ 1.67	<
Trachea	<	2.67	≤ 2.00	<	≤ 1.67	<	<	<	<	<	≤ 1.67	<
Right bronchus	≤ 1.67	2.67	≤ 2.50 ^h	<	<	<	<	<	<	<	<	3.00
Left bronchus	2.67	2.67	<	<	<	<	<	<	<	<	<	≤ 1.83
Right upper lung	<	<	<	<	<	<	≤ 3.17 ^j	<	<	<	<	<
Right middle lung	≤ 1.67	≤ 2.33 ^g	<	<	<	<	<	<	4.50	<	<	≤ 3.00 ⁱ
Right lower lung	<	≤ 2.33	<	<	<	<	≤ 2.33	≤ 1.83	<	<	<	<
Left upper lung	<	≤ 2.33	<	<	<	<	<	2.00	≤ 1.67	<	<	≤ 1.67
Left middle lung	<	<	<	<	<	<	3.67	≤ 2.23 ^f	<	<	<	≤ 1.83
Left lower lung	2.50	<	<	<	<	<	≤ 2.50	4.00	<	<	<	<

^a: Macaque identification.

^b<: No cytopathic effect (CPE)-positive well in quadruplicate culture. A detection limit is 1.67 \log_{10} TCID₅₀/g tissue.

^c ≤ 1.67 : One CPE-positive well in quadruplicate culture with undiluted samples was observed.

^d ≤ 1.83 : Two CPE-positive wells were observed in quadruplicate culture: one with undiluted samples and one with 10-fold diluted samples.

^e ≤ 2.00 : Two CPE-positive wells in quadruplicate culture with undiluted samples were observed.

^f ≤ 2.23 : Two and one CPE-positive wells were observed in quadruplicate culture of undiluted and 10-fold diluted samples, respectively.

^g ≤ 2.33 : Three CPE-positive wells in quadruplicate culture with undiluted samples were observed.

^h ≤ 2.50 : Four CPE-positive wells were observed in quadruplicate culture: two with undiluted samples and two with 10-fold diluted samples.

ⁱ ≤ 3.00 : Two and three CPE-positive wells were observed in quadruplicate culture of undiluted and 10-fold diluted samples, respectively.

^j ≤ 3.17 : Six CPE-positive wells were observed in quadruplicate culture: three with undiluted samples and three with 10-fold diluted samples.

Table S3. Clinical scoring

Parameter	Degree of parameter	Possible score
Fever	Normal (< 39 °C)	0
	Elevated temperature (39-40 °C)	3
	High temperature (> 40 °C)	5
Posture	Piloerection of body hair	1
	Decreased activity, decreasing normal behavior/Occasionally lying down, huddled, active when people in room	2
	Huddled on camera, active when people in room/Lying down, getting up when approached, using cage for support	3
	Huddled when people in room, shaking, toes and hands clenched/Lying down, not getting up when approached or prompted	5
Respiration	Increased or decreased; mild cough and clear nasal discharge	3
	Labored breathing through mouth; severe cough and severe nasal discharge	5
Appetite	Slightly decreased	1
	Decreased	2
	Severely decreased	5
Skin	Flushed appearance	2
	Visible rash	2
	Bleeding	5

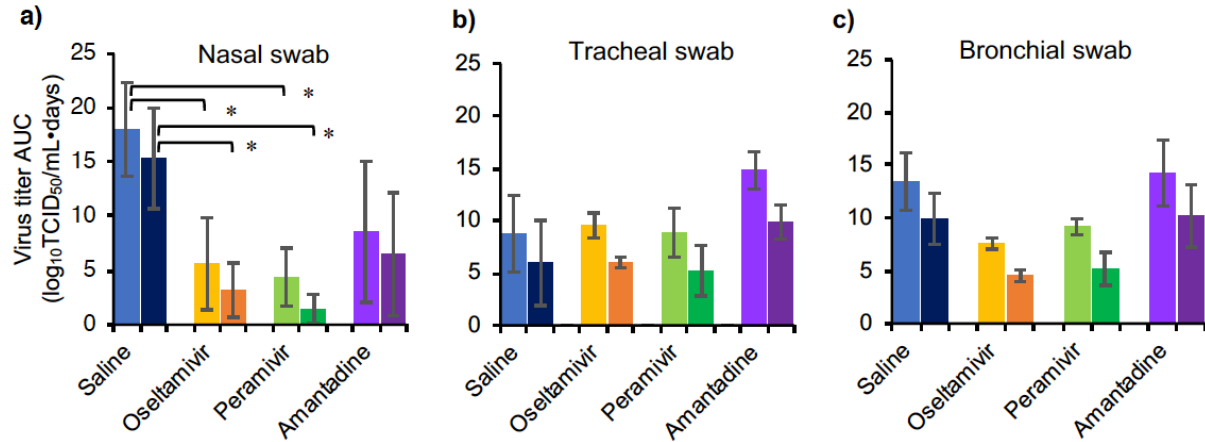


Fig S1. Virus titer AUCs in swab samples collected from cynomolgus macaques challenged with A/black swan/Akita/1/2016 (H5N6). The virus titer areas under the concentration-time curves (AUC, the summation of virus titers) of individual macaques were calculated on the basis of results in Table 1. The detection limit of virus titers in swab samples was 0.67 log₁₀TCID₅₀/mL. Virus titer AUCs of the virus titers (log₁₀TCID₅₀/mL) from days 1 to day 7 and from day 2 to day 7 were calculated by use of the trapezoidal rule. Thereafter, averages and standard deviations of the virus titer AUC of three macaques in each group were calculated. Left bars: AUC from day 1 to day 7 (including before treatment). Right bars: AUC from day 2 to day 7 (after treatment). The result of no CPE positive detection was counted as 0. When the result is lower than a certain number (for example, ≤ 0.67), we took that number (0.67) as the value of the virus titer, then analyzed data and drew the graphs. *: P < 0.05 (ANOVA multi-comparison test).

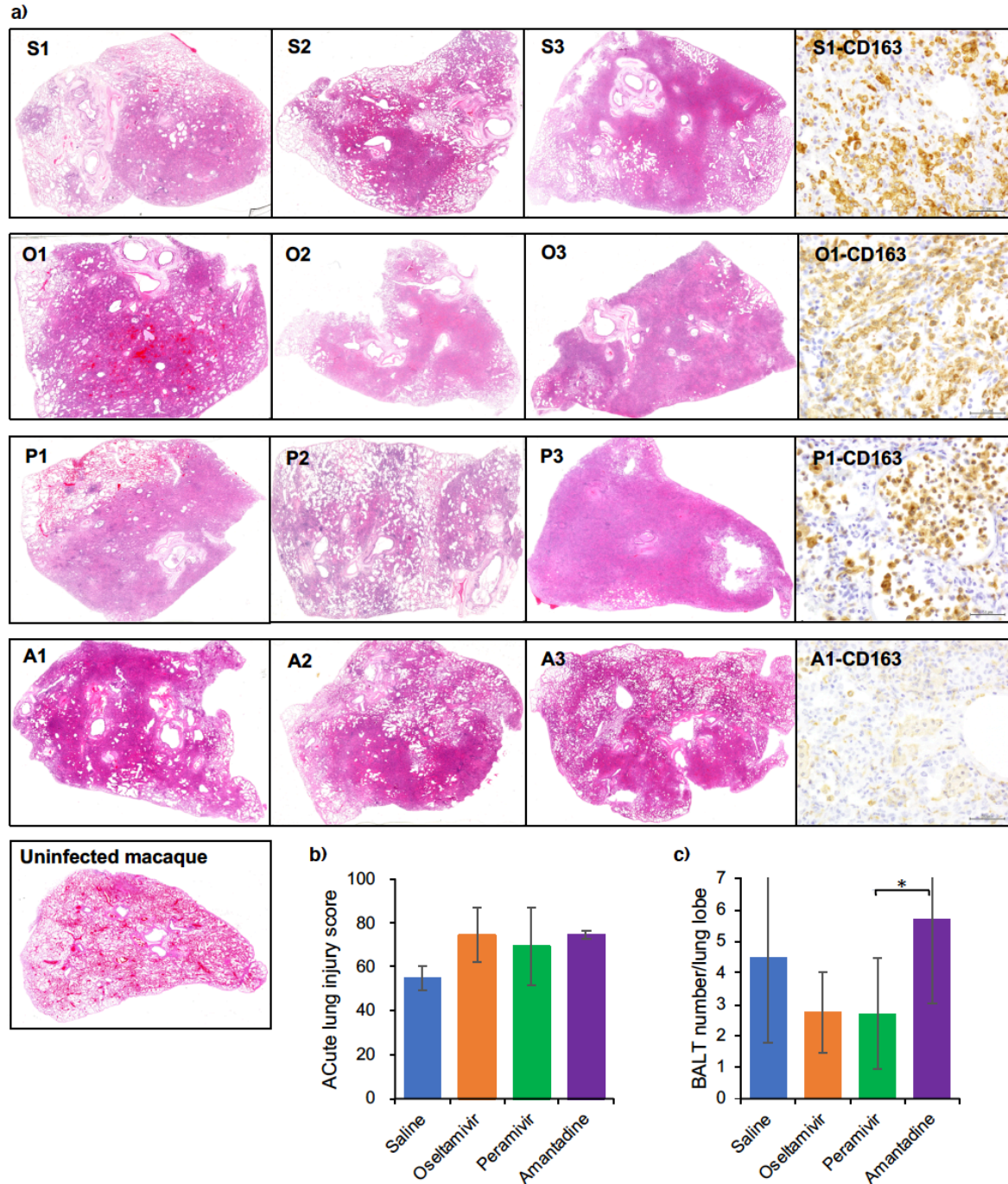


Fig S2. Pneumonia of cynomolgus macaques challenged with A/black swan/Akita/1/2016 (H5N6). (a) H&E sections of right lower lung lobes of 12 cynomolgus macaques and an uninfected macaque are shown. Images on the right column: immunohistochemical staining for CD163 (representative images of one macaque in each group). Bars, 50 μ m. (b) Averages of acute lung injury (ALI) scores. ALI scores were assessed by two pathologists who were blind to the macaque identification number. There is no significant difference in ALI scores among four groups. (c) The number of BALTs was counted on H&E staining sections of each lung lobe of macaques. The average BAL T number/lobe was used to compare among the groups. *: $P = 0.053$ (ANOVA multi-comparison test).

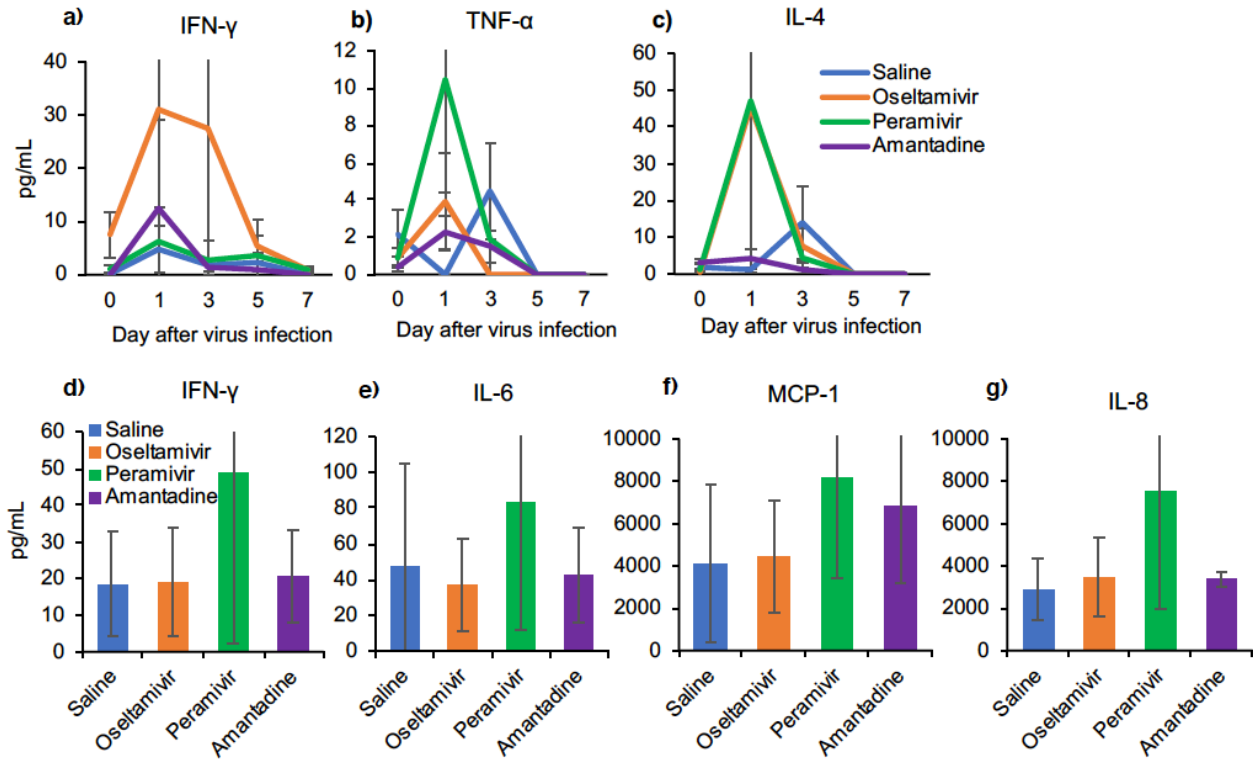


Fig S3. Levels of plasma and lung tissue cytokines in cynomolgus macaques challenged with A/black swan/Akita/1/2016 (H5N6). (a-c) The averages and standard deviations of cytokine concentrations in plasma after virus infection measured by a bead array assay. (a) IFN- γ , (b) TNF- α , (c) IL-4. (d-f) The concentrations of cytokines/chemokines in the lung tissues on day 7. The lung tissues were homogenized to prepare 10% w/v solution. (d) IFN- γ , (e) IL-6, (f) MCP-1, (g) IL-8.

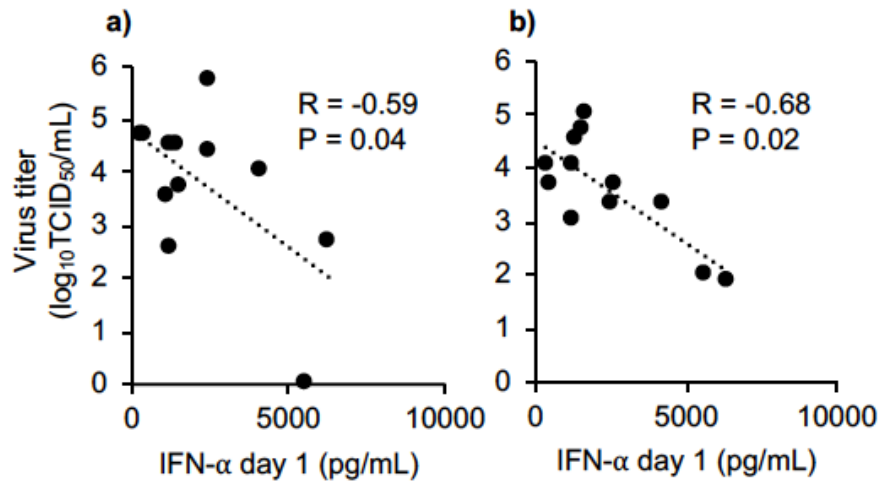


Fig S4. Correlation between IFN- α and virus titers in cynomolgus macaques after H5N6 virus infection. (a, b) Correlations of IFN- α concentration on day 1 with virus titers in the trachea (a) and bronchus (b) on day 1. R: correlation coefficient value. P: *p-value*. Statistics method: Pearson's product-moment correlation.

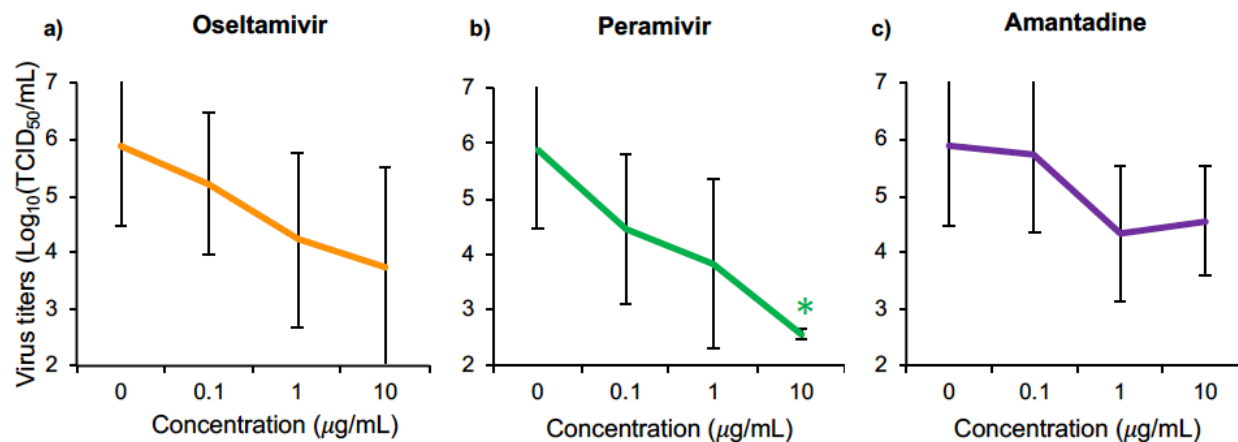


Fig. S5. Efficacy of antiviral drugs against A/Aichi/2/1968 (H3N2) virus *in vitro*.

MDCK cells were infected with the virus at a multiplicity of infection (MOI) of 0.01 and cultured with antiviral drugs of various concentrations: (a) oseltamivir, (b) peramivir, and (c) amantadine. The supernatant of each well was collected at 24 h after virus infection. Then virus titers in the supernatants were determined by the Reed Muench method. Averages and standard deviations of three independent experiments were shown. The asterisk shows a significant difference in virus titers between with and without antiviral drugs (Student's t-test, *: $P < 0.05$).