Comparative Inhibitory Effects of Various Nucleoside and Nonnucleoside Analogues on Replication of Influenza Virus Types A and B In Vitro and In Ovo

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Six nucleoside analogues, two sulfated polysaccharides, and four protease inhibitors were evaluated in vitro as inhibitors of influenza virus replication. Four guanosine analogues (mizoribine, ribavirin, pyrazofurin, and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide), the sulfated polysaccharide dextran sulfate (molecular weight 500,000), and two protease inhibitors (camostat mesilate and nafamostat mesilate) were inhibitory to the replication of strains of influenza virus types A and B at concentrations down to 0.3 μg/mL. Of these seven compounds, ribavirin, camostat mesilate, and nafamostat mesilate were efficacious in both reducing the virus titer and increasing the survival rate of influenza virus–infected chick embryos. For camostat mesilate, the ED_{50} (required to improve the survival rate of influenza virus–infected chick embryos by 50%) was 0.80 μg/g, and its selectivity index, based on the ratio of the 50% toxic dose (required to reduce the viability of chick embryos by 50%) to ED_{50}, was 280. Camostat mesilate deserves further exploration for its potential in the treatment of influenza virus infection.

The control of influenza virus epidemics has been a major health problem for many decades. Some compounds have been introduced for the treatment of influenza virus infection. Amantadine and rimantadine, which are used in the prophylaxis or treatment of early-stage infection, suffer from a narrow activity spectrum in that they are active against influenza virus type A but not type B infection [1, 2]. Ribavirin has been evaluated for its therapeutic activities against respiratory virus infections such as influenza virus, parainfluenza virus, and respiratory syncytial virus. However, the results of clinical trials with oral ribavirin in patients with influenza virus infection have not been encouraging [3, 4]. Thus, there is a need for compounds that are potent and selective inhibitors of both influenza virus types A and B.

We have recently proved that several compounds, such as the nucleoside analogues pyrazofurin [5] and 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) [6] and sulfated polysaccharides [7], inhibit the appearance of virus-induced cytopathic effect in MDCK cells infected with laboratory strains of influenza virus. In this study, six nucleoside analogues and two sulfated polysaccharides were compared with four protease inhibitors [8] for effect on the protection of cells infected with strains of influenza virus types A and B, including several clinical strains from virus-induced destruction, and for toxicity to host cells. Of these compounds, seven were also evaluated for their inhibitory effects on growth of influenza virus in vitro, in cell culture, and in ovo in chick embryos.

Materials and Methods

Compounds. The test compounds and their sources were as follows: ribavirin (relative molecular weight [M_r] 244; ICN Pharmaceuticals, Costa Mesa, CA), EICAR (M_r 267; from A. Matsuda, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan), pyrazofurin (M_r 259; Calbiochem-Behring, Lucerne, Switzerland), mizoribine (M_r 259; Toyo-Jozo, Shizuoka, Japan), neplanocin A (M_r 263; Toyo-Jozo), 6′-*C*-(*R*)-methylneplanocin A (M_r 277; Toyo-Jozo), dextran sulfate (M_r 5000 and 500,000; Sigma, St. Louis), aprotinin (M_r 6512; Sigma), gabexate mesilate (M_r 417; Ono Pharmaceutical, Osaka, Japan), camostat mesilate (M_r 495; Ono), and nafamostat mesilate (M_r 540; Torii Pharmaceutical, Osaka).

Viruses. Four influenza virus type A strains and 4 type B strains were used in the experiments. Of these, 3 were laboratory strains: A/Ishikawa/7/82 (H3N2), A/Victoria/3/75 (H3N2), and B/Singapore/222/79. The other 5 strains were clinical [5]: A/Fukushima/88/84 (H1N1), A/Fukushima/89/84 (H1N1), B/Fukushima/274/85, B/Fukushima/275/85, and B/Fukushima/276/85. Virus-infected cells were frozen at −80°C, thawed at 37°C, and then centrifuged at 1600 *g* for 10 min. After centrifugation, the supernatant was used as the stock virus.

Antiviral activity assay in vitro. Inhibition of virus-induced cytopathogenicity was measured by modifications of a tetrazolium-based method [9]. Confluent MDCK cells were grown in a 96-well microtiter tray in growth medium that consisted of Eagle MEM supplemented with 10% fetal calf serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin. After the growth medium was withdrawn, each well was inoculated with 100 μL of serial dilutions of the test compounds and 100 μL of virus suspension containing 100 times the $TCID_{50}$. The maintenance medium consisted of Eagle MEM supplemented with 1% fetal

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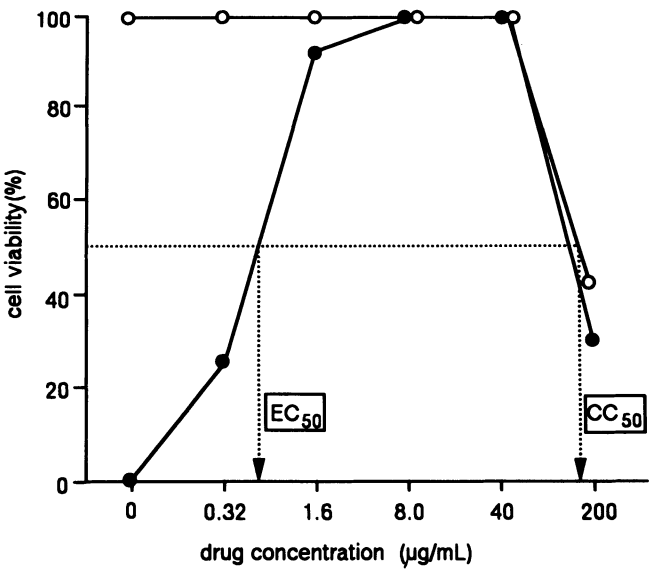

Figure 1. Antiviral activity and cytotoxicity of test compounds were measured by modifications of tetrazolium-based method. EC_{50} was concentration that protected 50% of virus-infected cells (●) from virus-induced destruction; CC_{50} was concentration required to reduce viability of mock-infected cells (○) by 50%. Test compound was nafamostat mesilate.

calf serum and antibiotics. No trypsin was added in any of the experiments. After 4–6 days of incubation at 35°C, when MDCK cells in control cultures were completely destroyed as the consequence of viral replication, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide (MTT) solution (7.5 mg/mL) in PBS was added to each well of the microtiter tray. The tray was then incubated at 37°C for 2 h; 170 μL of medium was removed from each well without disturbing the cell cultures, and 100 μL of acidified isopropanol (2 mL of concentrated HCl/500 mL of isopropanol) containing 10% (vol/vol) Triton X-100 was added to each well to solubilize the formazan crystals. After the tray was shaken for 10 min to completely solubilize the formazan crystals, the A_{540} and A_{690} of the wells were read in a computer-controlled microplate reader (model 3550; Bio-Rad, Richmond, CA). The 50% effective antiviral concentration (EC_{50}) was expressed as the concentration that protected 50% of the cells from virus-induced destruction (figure 1).

Cytotoxicity and cytostatic activity. The concentration required to reduce the viability of cells by 50%, as measured by the MTT method, was estimated and is described as the 50% cytotoxic concentration (CC_{50}; figure 1). Inhibition of the proliferation of MDCK cells was monitored by counting the number of viable cells (after staining with trypan blue). The concentration required to inhibit cell proliferation by 50% was estimated as the 50% inhibitory concentration (IC_{50}).

Inhibition of viral yield in vitro. MDCK cells were seeded in 24-well tissue culture trays. When confluent, the cells were washed once with maintenance medium and infected with 1000 times the $TCID_{50}$ of influenza virus type A (strain Ishikawa) in 0.5 mL of maintenance medium. After virus adsorption at 35°C for 1 h, the cells were washed twice with maintenance medium, and the medium was replaced with new medium containing the test compound at 2- or 10-fold higher concentration than its EC_{50}. Immediately after virus adsorption (time 0) and after incubation for 12, 24, 48, and 72 h at 35°C, the culture medium was collected as a test sample and stored at −80°C until titra-

Table 1. Inhibitory effects of test compounds on replication of influenza virus types A and B in vitro.

Compound	EC_{50} for type A virus				Average for type A	EC_{50} for type B virus				Average for type B
	Ishikawa	Victoria	Fu-88	Fu-89		Singapore	Fu-274	Fu-275	Fu-276	
Guanosine analogues										
Mizoribine	14	8.5	6.4	12	10 (39)	6.7	3.6	15	8.9	8.6 (33)
Ribavirin	3.6	5.2	4.0	5.0	4.5 (18)	3.8	1.3	3.1	3.5	2.9 (12)
Pyrazofurin	2.0	0.55	0.38	1.4	1.1 (4.2)	0.71	0.73	1.2	0.58	0.81 (3.1)
EICAR	1.4	0.92	0.30	0.45	0.77 (2.9)	0.87	0.16	0.43	0.41	0.47 (1.8)
Adenosine analogues										
Neplanocin A	>28	—	—	—	—	>28	—	—	—	—
6′-*C*-(*R*)-methyneplanocin A	>200	—	—	—	—	>200	—	—	—	—
Dextran sulfate										
M_r 5000	>200	—	—	—	—	>200	—	—	—	—
M_r 500,000	4.9	3.6	15	7.3	7.7 (0.015)	28	3.6	4.9	8.4	11 (0.022)
Protease inhibitors										
Aprotinin	>200	—	—	—	—	>200	—	—	—	—
Gabexate mesilate	>200	—	—	—	—	>200	—	—	—	—
Camostat mesilate	2.2*	2.0	3.5	5.1	3.2 (6.5)	5.8*	2.0	2.6	3.9	3.6 (7.3)
Nafamostat mesilate	0.44*	0.55	0.76	0.80	0.64 (1.2)	1.5*	0.57	0.70	0.48	0.81 (1.5)

NOTE. Data are 50% effective concentration (EC_{50}) in μg/mL; nos. in parentheses are μ*M*. Data are mean of 2 or 3 experiments. EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; M_r, relative molecular weight; Fu-88, A/Fukushima/88/84; Fu-89, A/Fukushima/89/84; Fu-274, B/Fukushima/274/85; Fu-275, B/Fukushima/275/85; Fu-276, B/Fukushima/276/85.

* From [8].

Table 2. In vitro selectivity of test compounds as inhibitors of influenza virus type A replication.

Compound	EC_{50}	CC_{50}	IC_{50}	Selectivity index CC_{50}/EC_{50}	Selectivity index IC_{50}/EC_{50}
Guanosine analogues					
Mizoribine	10	>200	1.7	>20	0.17
Ribavirin	4.5	>200	7.5	>44	1.7
Pyrazofurin	1.1	16	0.44	15	0.40
EICAR	0.77	>200	0.34	>260	0.44
Adenosine analogues					
Neplanocin A	>28	28	—	<1	
6′-*C*-(*R*)-methylneplanocin A	>200	>200	—	><1	
Dextran sulfate					
M_r 5000	>200	>200	—	><1	
M_r 500,000	7.7	>200	>1000	>26	>130
Protease inhibitors					
Aprotinin	>200	>200	—	><1	
Gabexate mesilate	>200	>200	—	><1	
Camostat mesilate	3.2	>200	>1000	>62	>310
Nafamostat mesilate	0.64	150	76	230	120

NOTE. 50% effective concentration (EC_{50}), 50% cytotoxic concentration (CC_{50}), and 50% inhibitory concentration (IC_{50}) are given in μg/mL. EC_{50}, average for all type A strains; CC_{50} and IC_{50}, means of 2 experiments. EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; M_r, relative molecular weight.

tion. The samples thus obtained were titrated simultaneously for infectivity in microtiter trays of MDCK cell culture.

Antiviral activity assay in ovo. Serial dilutions (0.5 mL) of the test compounds were injected into the allantoic cavity of 8-day-old chick embryos. Ten chick embryos were used for each treated group. At 30 min after injection, the allantoic cavities were infected with 100 times the $TCID_{50}$ of influenza virus type A (strain Ishikawa). Embryos were incubated at 37°C for 72 h. Then survival rate and mean HA (hemagglutinin) titer (using 1% human erythrocytes) of allantoic fluid for each group were assessed. The dose required to increase the survival rate of virus-infected chick embryos by 50% was estimated as the ED_{50}. The 90% inhibitory dose (ID_{90}) was estimated as the dose that reduced the HA titer by 90%.

Toxicity in ovo. Serial dilutions (0.5 mL) of the test compounds were injected into the allantoic cavity of 8-day-old chick embryos. After 72 h of incubation at 37°C, the survival rate for each group was assessed. The dose required to reduce the survival rate by 50% was estimated as the 50% toxic dose (TD_{50}).

Results

The in vitro antiviral activities of the test compounds were assessed on the basis of their ability to protect cells infected with strains of influenza virus types A and B from virus-induced destruction (table 1). Four nucleoside analogues (mizoribine, ribavirin, pyrazofurin, and EICAR), dextran sulfate (M_r 500,000), and two protease inhibitors (camostat mesilate and nafamostat mesilate) inhibited the replication of different influenza virus type A and B strains at concentrations of 0.3–28.0 μg/mL. Neplanocin A, 6′-*C*-(*R*)-methylneplanocin A, dextran sulfate (M_r 5000), aprotinin, and gabexate mesilate showed no activity against influenza viruses at the highest concentrations tested.

When the cytotoxicity was evaluated by the reduction of cell viability (CC_{50}; table 2), pyrazofurin, neplanocin A, and nafamostat mesilate were toxic to host cells at concentrations of 16, 28, and 150 μg/mL, respectively, whereas the other compounds were not toxic to MDCK cells at 200 μg/mL. In the next set of experiments, the selected test compounds were examined for their inhibitory effects on cell proliferation (IC_{50}; table 2). The IC_{50}s of guanosine analogues were 5-fold lower to 2-fold higher than the corresponding EC_{50}s. Dextran sulfate and camostat mesilate showed no cytostatic activity at concentrations up to 1000 μg/mL. Nafamostat mesilate inhibited cell proliferation at an IC_{50} that was 2-fold lower than the CC_{50}. Marked selectivity ($IC_{50}/EC_{50} \gg 1$; table 2) was observed for only three compounds (dextran sulfate [M_r 500,000], camostat mesilate, and nafamostat mesilate).

The selected compounds were examined for their inhibitory effects on the growth of influenza virus in cell culture (figure 2). All compounds inhibited growth compared with control virus growth. At 72 h after infection, the virus yield in the cell culture exposed to the test compound at a concentration 10 times higher than EC_{50} was $\sim 10^2$- to 10^4-fold lower than the control virus yield. The order of inhibitory effect of test compounds was as follows: ribavirin = nafamostat mesilate > camostat mesilate > pyrazofurin > dextran sulfate > EICAR = mizoribine.

Figure 3A shows the anti-influenza virus type A activity of ribavirin in ovo. Ribavirin reduced the HA titer partially at 4 μg/g and completely at 20 and 100 μg/g. At these doses, ribavirin also effected 100% survival. At 500 μg/g, ribavirin proved to be 100% lethal for the chick embryos. Dextran sulfate (M_r 500,000; figure 3B) partially reduced the HA titer but did not raise the survival rate to more than one-third. Camostat mesilate (figure 3C) brought about a dose-dependent decrease in HA titer within the dose range of 0.8–100 μg/g. At 4, 20, and 100 μg/g, camostat mesilate completely protected virus-infected chick embryos from death. The results of the in ovo studies are summarized in table 3. Three compounds (ribavirin, camostat mesilate, and nafamostat mesilate) were effective in both reducing the HA titer and increasing the survival rate of virus-infected chick embryos. Three other compounds (mizoribine, pyrazofurin, and EICAR) did not reduce the HA titer, nor did they reduce the mortality rate of the virus-infected embryos. The selectivity indexes, based on the ratio of TD_{50} to ED_{50}, of ribavirin, camostat mesilate, and nafamostat mesilate were 55, 280, and 28, respectively.

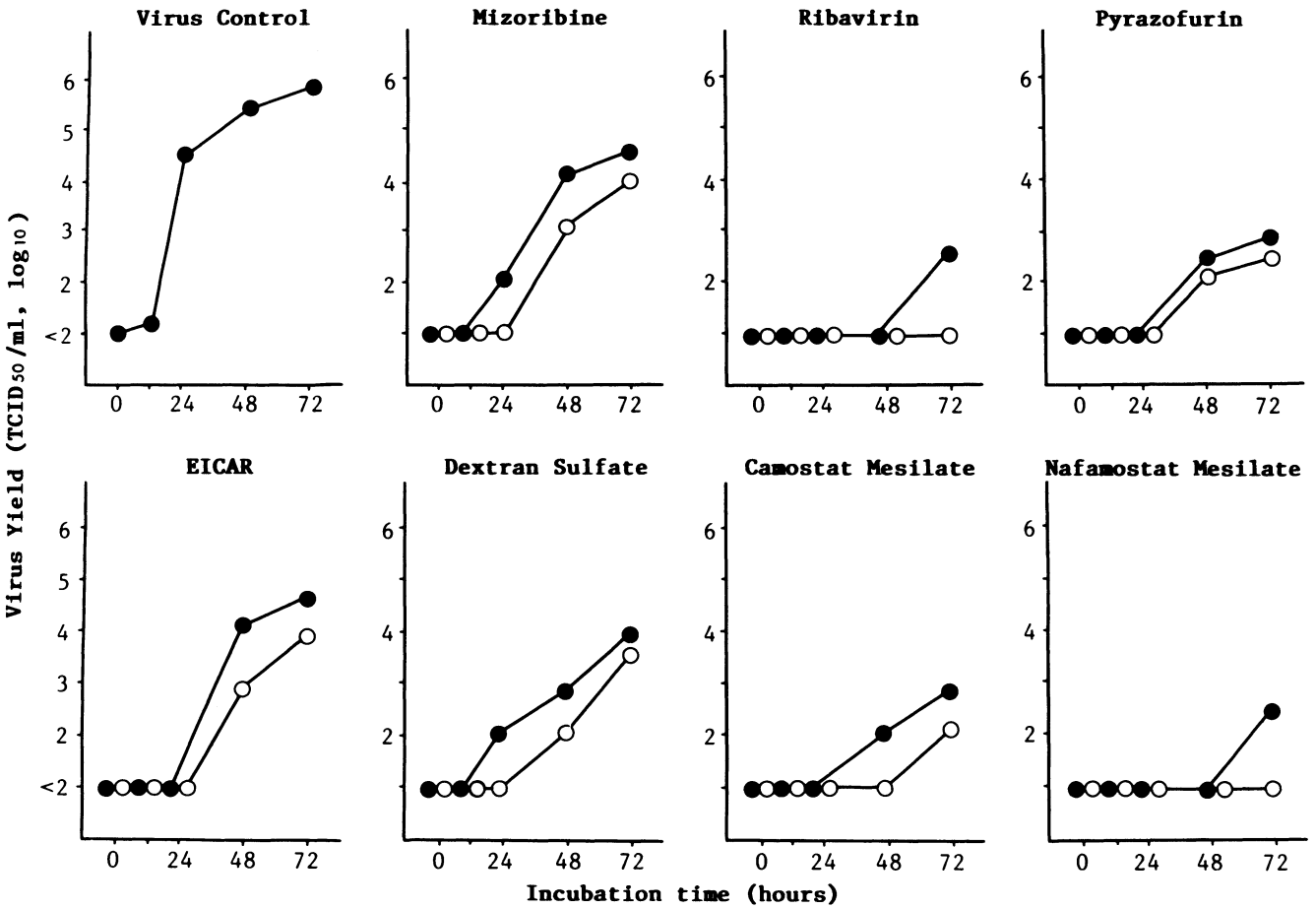

Figure 2. Growth curves of influenza virus in MDCK cells treated with antiviral compounds. EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; dextran sulfate had molecular weight of 500,000. Cells were infected with influenza virus type A (strain Ishikawa) at MOI of 0.01. After virus adsorption, monolayers were washed twice with maintenance medium, exposed to test compounds at concentration 2 (●) or 10 times (○) 50% effective concentration, and further incubated at 35°C. At indicated times, cell culture medium was collected, stored at −80°C, and titrated for infectious virus.

Discussion

The guanosine analogues mizoribine, ribavirin, pyrazofurin, and EICAR inhibited influenza virus replication in vitro. Mizoribine [10], which is used clinically as an immunosuppressive drug, inhibits inosine monophosphate dehydrogenase (IMPD) and guanosine monophosphate synthetase and thus blocks lymphocyte proliferation and viral replication. IMPD is also assumed to be the main target for the antiviral action of EICAR [6] and ribavirin [11]. Orotidine monophosphate decarboxylase is assumed to be the target enzyme for pyrazofurin [12]. Mizoribine, EICAR, pyrazofurin, and ribavirin were inhibitory to influenza viruses in vitro, but of these four nucleoside analogues, only ribavirin increased the survival rate of influenza virus–infected chick embryos. These observations suggest that compounds that interfere with an enzymatic step involved in nucleotide biosynthesis may inhibit viral replication in vitro in cell culture but not in ovo in chick embryos. The effectiveness of ribavirin in ovo may well depend on mechanism(s) other than IMPD inhibition, such as inhibition of influenza virus RNA polymerase [13, 14]. The S-adenosylhomocysteine hydrolase inhibitors neplanocin A and 6′-*C*-(*R*)-methylneplanocin A are apparently inhibitory to paramyxoviruses (e.g., respiratory syncytial virus, measles virus) [15] but not to influenza virus.

Dextran sulfate is inhibitory to various enveloped viruses in vitro [16, 17]. The mechanism of anti–influenza virus action of polyanions was attributed to inhibition of the viral envelope–cell membrane fusion process [7, 18]. We found that dextran sulfate (M_r 500,000) inhibited influenza virus types A and B in vitro. It partially reduced the HA titer in the allantoic fluid but did not markedly increase the survival rate of the infected chick embryos.

Protease inhibitors such as camostat mesilate and nafamostat mesilate inhibit the replication of influenza virus types A and B by blocking the cleavage of viral HA glycoprotein [8, 19]. The protease inhibitors not only suppressed the

replication of influenza virus in vitro and in ovo but also protected the chick embryos from virus-induced mortality without being themselves toxic to the host. In particular, the antiviral selectivity of camostat mesilate in ovo seemed to be superior to that of ribavirin. This result suggests that the protease that activates influenza virus HA glycoprotein may be a good target for anti-influenza virus drugs.

On the basis of our in vitro and in ovo data, we postulate that protease inhibitors such as camostat mesilate and nafamostat mesilate should be further pursued for their potential use in the prophylaxis and therapy of influenza virus (type A and B) infections.

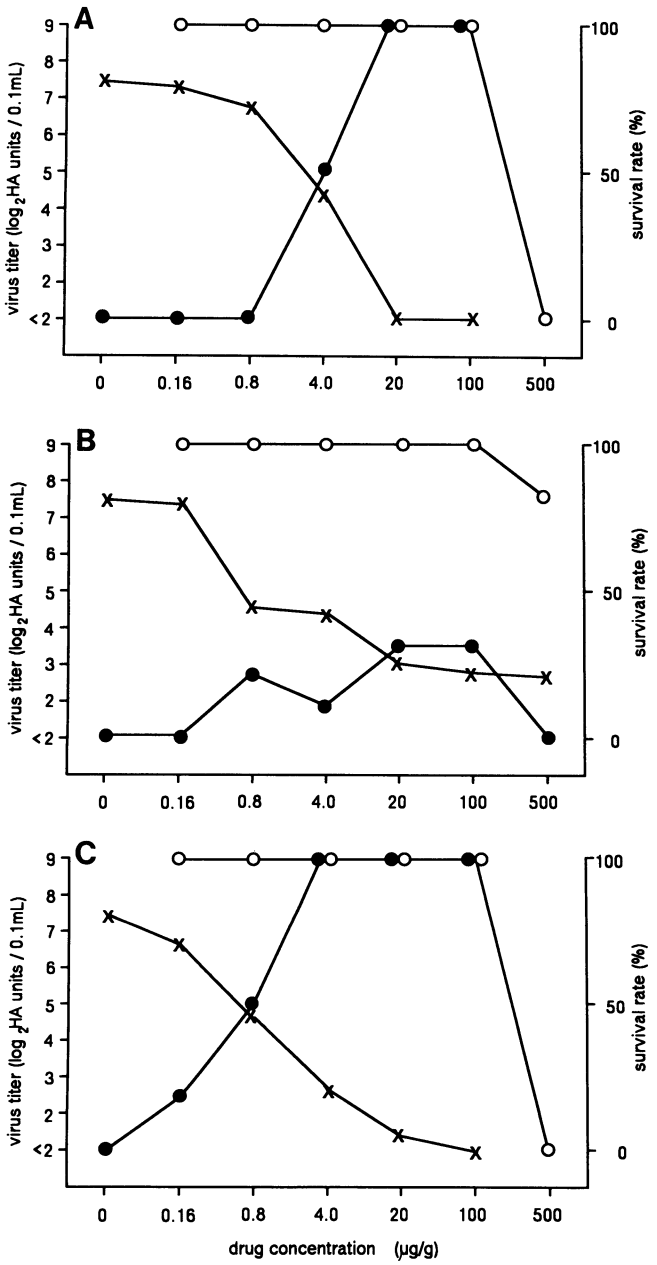

Figure 3. 8-day-old chick embryos were injected with test compounds; then they were infected with 100 times $TCID_{50}$ of influenza virus type A (strain Ishikawa). At 72 h, mean HA titer of allantoic fluid (X) and survival rate (●) for each group were assessed. Toxicity was estimated from survival rate of treated mock-infected chick embryos (○). Test compounds were ribavirin (**A**), dextran sulfate with molecular weight of 500,000 (**B**), and camostat mesilate (**C**).

Table 3. In ovo selectivity of test compounds as inhibitors of influenza virus type A replication.

Compound	ID_{90}	ED_{50}	TD_{50}	Selectivity index TD_{50}/ID_{90}	Selectivity index TD_{50}/ED_{50}
Guanosine analogues					
Mizoribine	>130	>130	130	<1	<1
Ribavirin	4.6	4.0	220	48	55
Pyrazofurin	>68	>68	68	<1	<1
EICAR	>10	>10	10	<1	<1
Dextran sulfate (M_r 500,000)	6.0	>500	>500	>83	><1
Protease inhibitors					
Camostat mesilate	1.2	0.80	220	180	280
Nafamostat mesilate	1.1	1.8	50	45	28

NOTE. 90% inhibitor dose (ID_{90}), 50% effective dose (ED_{50}), and 50% toxic dose (TD_{50}) are given in μg/g. EICAR, 5-ethynyl-1β-D-ribofuranosylimidazole-4-carboxamide.

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