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Effects of Protease Inhibitors on Replication of Various Myxoviruses

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We studied the effects of eight protease inhibitors on the multicycle replications of various orthomyxoviruses and paramyxoviruses. Among the compounds, nafamostat mesilate, camostat mesilate, gabexate mesilate, and aprotinin, which are widely used in the treatment of pancreatitis, inhibited influenza virus A and B replication at concentrations that were significantly lower than their cytotoxic thresholds in vitro. None of the protease inhibitors had activity against respiratory syncytial virus, measles virus, or parainfluenza virus type 3 at the highest concentrations tested. Camostat mesilate was found to be the most selective inhibitor. Its 50% effective concentration for influenza virus A replication was 2.2 μg/ml, and the selectivity index, which was based on the ratio of the 50% inhibitory concentration for host cell proliferation to the 50% effective concentration for influenza virus A replication, was 680. When the in ovo antiviral activity of the compounds was tested by using chicken embryos, camostat mesilate at a dose of 10 μg/g markedly reduced the hemagglutinin titers of influenza viruses A and B.

The myxoviruses (orthomyxoviruses and paramyxoviruses), like other enveloped viruses, contain glycoproteins which are located outside the virion lipid bilayers. The hemagglutinin (HA) of influenza viruses and the fusion protein of paramyxoviruses undergo posttranslational proteolytic cleavage. Since the cleavage must precede the fusion process, cleavage is essential for the infectivity and spread of the virus in the host organism. The cleavage of these viral glycoproteins is accomplished by the host cellular protease (9–11, 15). Recently, it was shown that protease inhibitors such as aprotinin (21) and nafamostat mesilate (17) inhibit the replication of influenza virus A by blocking the cleavage of viral glycoprotein. We evaluated the effects of several protease inhibitors on the multicycle replication of various orthomyxoviruses and paramyxoviruses in vitro and in ovo.

MATERIALS AND METHODS

Viruses. The following viruses were used in the experiments described here: influenza virus [A/Ishikawa/7/82(H3N2) strain and B/Singapore/222/79 strain] (16), respiratory syncytial virus (RSV) (Long strain) (8), measles virus (Sugiyama strain) (7), parainfluenza virus type 3 (HA-1, C243 strain) (12), and parainfluenza virus type 1 (HVJ, Fushimi strain).

Compounds. Camostat mesilate (Foipan) and gabexate mesilate (FOY) were generous gifts from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Trypsin-chymotrypsin inhibitor, pepstatin A, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nafamostat mesilate (Futhan; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), aprotinin (Trasylol; Bayer Yakuhin, Ltd., Osaka, Japan), ulinastatin (Miraclid; Mochida Fine Chemicals Industry Ltd., Tokyo, Japan), and ribavirin (Virazole; ICN Nutritional Biochemicals, Cleveland, Ohio) were used. The chemical structures of the protease inhibitors are shown in Fig. 1.

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Antiviral activity in vitro. Inhibition of virus-induced cytopathogenicity was measured by modifications of a tetrazolium-based method (13). Confluent cells (MDCK cells for influenza viruses A and B, HeLa cells for RSV, and Vero cells for measles virus and parainfluenza virus type 3) were grown in a 96-well microtiter tray in growth medium that consisted of Eagle's minimum essential medium supplemented with 10% fetal calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. The growth medium was withdrawn, and each well was inoculated with 100 μl of virus suspension containing 100 50% cell culture infectious doses and 100 μl of serial dilutions of the test compounds. The maintenance medium consisted of Eagle's minimum essential medium supplemented with 1% fetal calf serum and antibiotics. No trypsin was added in any of the experiments. After 4 days (for influenza virus B, measles virus, or

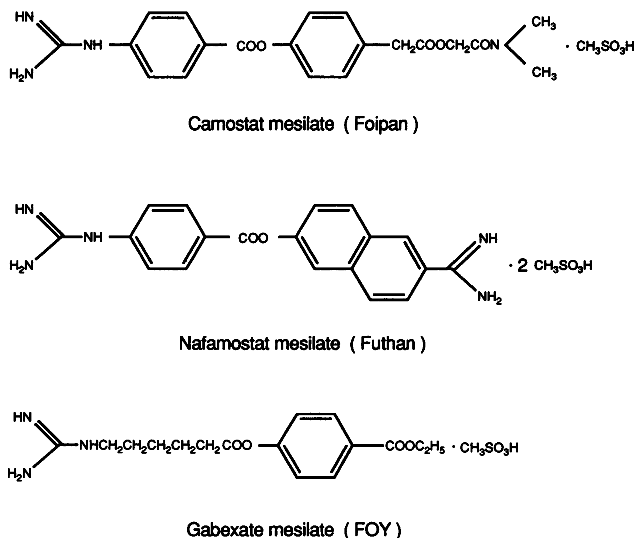


FIG. 1. Chemical structures of protease inhibitors.

TABLE 1. Effects of protease inhibitors on orthomyxoviruses and paramyxoviruses

Compound	EC_{50} (μg/ml)[a]				
	Influenza virus A	Influenza virus B	RSV	Measles virus	Parainfluenza virus type 3
Nafamostat mesilate	0.44 (0.32–0.58)	1.5 (1.2–1.7)	>36	>110	>110
Camostat mesilate	2.2 (1.4–3.2)	5.8 (3.7–8.0)	>500	>500	>500
Gabexate mesilate	230 (200–260)	130 (60–190)	>120	>150	>150
Aprotinin[b]	170 (22–360)	260 (180–340)	>500	>500	>500
Ulinastatin	>500	>500	>500	>500	>500
Leupeptin	>500	>500	>500	>500	>500
Pepstatin A	>500	>500	>500	>500	>500
Trypsin-chymotrypsin inhibitor	>500	>500	>500	>500	>500
Ribavirin	3.6 (3.2–4.0)	3.8 (2.2–5.4)	5.2 (3.8–6.6)	30 (21–39)	16 (12–20)

[a] EC_{50}, 50% effective concentration, or the concentration required to inhibit virus cytopathogenicity by 50%. Data are means, with ranges given in parentheses.
[b] For aprotinin, data are given in units per milliliter.

parainfluenza virus type 3) or 6 days (for RSV or influenza virus A) of incubation at 35°C, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (7.5 mg/ml) in phosphate-buffered saline was added to each well of the microtiter tray. The tray was then incubated at 37°C for 2 h. Approximately, 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 per 500 ml of isopropanol) containing 10% (vol/vol) Triton X-100 was added to each well to solubilize the formazan crystals. After shaking the tray for 10 min, whereby formazan crystals were solubilized completely, the A_{540} and A_{690} of the wells were read in a computer-controlled microplate reader (model 3550; Bio-Rad). The 50% effective antiviral concentration was expressed as the concentration that protected 50% of the cells from virus-induced destruction.

Cytotoxicity. 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 (6).

Cytostatic activity. Inhibition of the proliferation of MDCK cells was assessed during their exponential growth phase and was monitored by counting the number of viable cells (following staining with trypan blue) (3, 4). The concentration required to inhibit cell proliferation by 50% was estimated and is described as the 50% inhibitory concentration.

Antiviral activity in ovo. The allantoic cavities of 8-day-old chicken embryos were infected with 100 50% cell culture infectious doses of influenza virus A, influenza virus B, or parainfluenza virus type 1 per egg. At 30 min after infection, 0.5 ml of serial dilutions of test compounds was injected into the allantoic cavity. Embryos were incubated at 35°C for 48 h (for parainfluenza virus type 1) or 72 h (for influenza virus A or B). At the end of the incubation period, samples of allantoic fluid were collected, and their HA titers (using 1% human erythrocytes) were assessed. Four chicken embryos were used for each treated group.

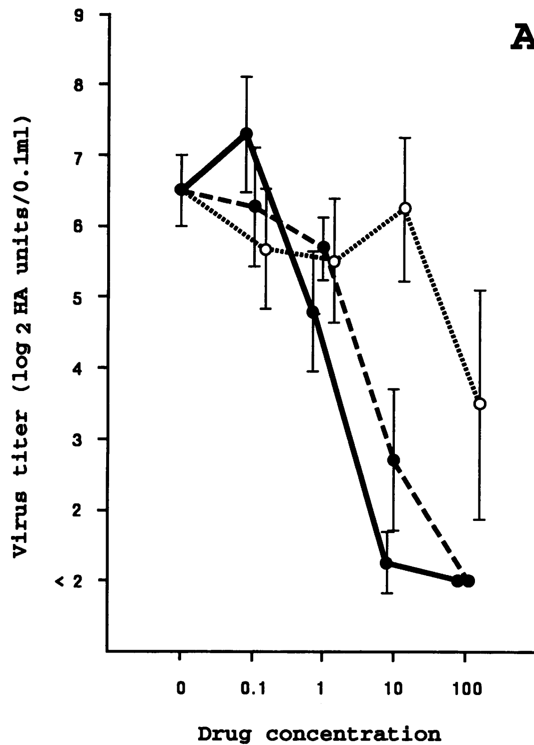
RESULTS

The in vitro antiviral activities of the protease inhibitors were assessed on the basis of their inhibitory effects on the cytopathogenicities of orthomyxoviruses and paramyxoviruses. Nafamostat mesilate, camostat mesilate, gabexate mesilate, and aprotinin were found to be inhibitory for influenza viruses A and B but not for RSV, measles virus, or parainfluenza virus type 3 (Table 1). Marked activity against influenza viruses was noted with nafamostat mesilate and camostat mesilate. The EC_{50}s of nafamostat mesilate for influenza viruses A and B were 0.44 and 1.5 μg/ml, respectively, and those of camostat mesilate were 2.2 and 5.8 μg/ml, respectively (Table 1).

TABLE 2. Selectivity effects of protease inhibitors against influenza virus A

Compound	CC_{50} (μg/ml)[a]			IC_{50} (μg/ml), MDCK cells[b]	SI (IC_{50}/EC_{50})[c]
	MDCK cells	HeLa cells	Vero cells		
Nafamostat mesilate	150	36	110	76	170
Camostat mesilate	>500	>500	>500	1,500	680
Gabexate mesilate	580	120	150	120	0.52
Aprotinin[d]	>500	>500	>500	>2,000	>12
Ulinastatin	>500	>500	>500	ND[e]	
Leupeptin	>500	>500	>500	ND	
Pepstatin A	>500	>500	>500	ND	
Trypsin-chymotrypsin inhibitor	>500	>500	>500	ND	
Ribavirin	>500	>500	>500	7.5	2.1

[a] CC_{50}, 50% cytotoxic concentration, or the concentration required to reduce cell viability by 50%, as determined by the MTT method. Values are means of two separate experiments.
[b] IC_{50}, 50% inhibitory concentration, or the concentration required to inhibit cell proliferation by 50%.
[c] SI is the ratio of the 50% inhibitory concentration for MDCK cell proliferation to the 50% effective antiviral concentration for influenza virus A replication.
[d] For aprotinin, data are given in units per milliliter.
[e] ND, not determined.



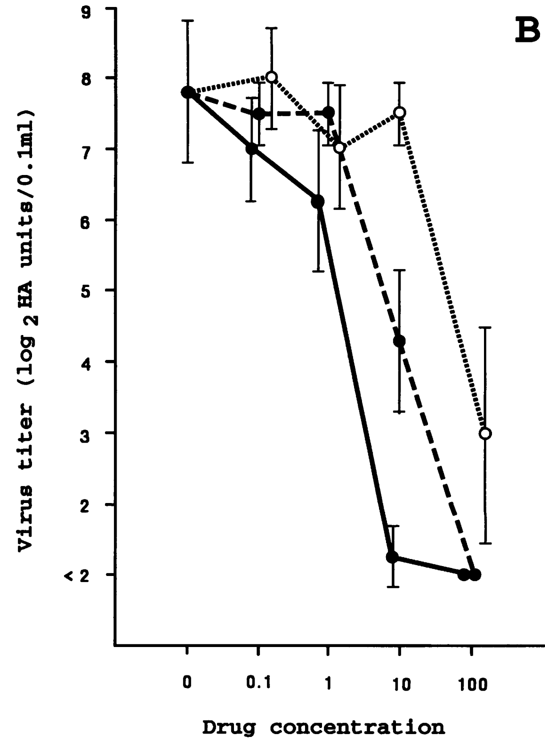
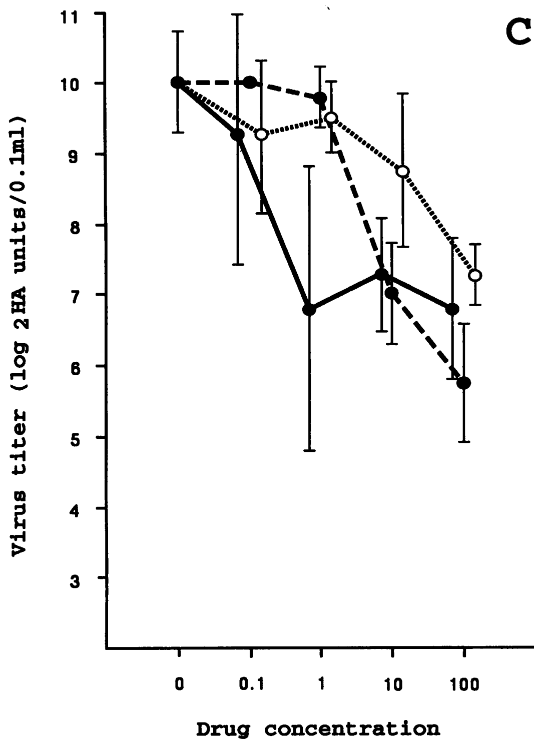


FIG. 2. Inhibitory effects of camostat mesilate (——, in micrograms per gram), ribavirin (– – –, in micrograms per gram), and aprotinin (· · · · , in units per gram) on the replication of influenza viruses A (A) and B (B) and parainfluenza virus type 1 (C) in chicken embryos.



When the cytotoxicity was evaluated by the reduction of cell viability (50% cytotoxic concentration), nafamostat mesilate was toxic to MDCK cells at a concentration of 150 μg/ml, whereas camostat mesilate and ribavirin were not toxic at the highest concentration tested (500 μg/ml) (Table 2). In the next set of experiments, the selected compounds were examined for their antiproliferative actions (50% inhibitory concentrations). Nafamostat mesilate, camostat mesilate, and ribavirin proved to be inhibitory to the growth of MDCK cells at concentrations of 76, 1,500, and 7.5 μg/ml, respectively (Table 2). The selectivity index (SI), which was based on the ratio of the 50% inhibitory concentration for host cell proliferation to the 50% effective antiviral concentration for influenza virus A replication, of camostat mesilate (SI = 680) was much higher than those of nafamostat mesilate (SI = 170) and ribavirin (SI = 2.1).

Figure 2 shows the antimyxoviral activities of the compounds in ovo. When administered at a dose of either 10 or 100 μg/g, camostat mesilate brought about a significant reduction in the HA titer of influenza viruses A and B without apparent toxicity to the host. This activity seemed to be superior to that of ribavirin. Also, camostat mesilate reduced considerably the HA titer of parainfluenza virus type 1 at doses of 1, 10, and 100 μg/g. Aprotinin, however, showed a slight inhibition on the replication of influenza viruses A and B and parainfluenza virus type 1 at the highest dose tested (100 U/g).

DISCUSSION

We examined eight protease inhibitors for their inhibitory effects on the replication of various orthomyxoviruses and paramyxoviruses in vitro and in ovo. Four of eight compounds inhibited influenza virus A and B replication at a lower concentration than that which showed toxicity to the host cells. Two compounds in particular (nafamostat mesilate and camostat mesilate) had remarkable activities. The differences in the activities of the compounds were sup-

ORTHOMYXOVIRUSES

Influenza virus type A
Influenza virus type B

PARAMYXOVIRUSES

Parainfluenza virus type 1
Parainfluenza virus type 3
Measles virus
Respiratory syncytial virus

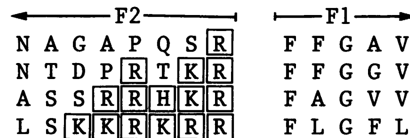


FIG. 3. Comparison of the amino acid sequences of the HA glycoprotein of orthomyxoviruses and the fusion glycoprotein of paramyxoviruses. Basic amino acids are boxed.

ported by statistical analysis ($P < 0.05$; Student's t test). The potencies of nafamostat mesilate and camostat mesilate were comparable to that of ribavirin. When we examined the cytotoxicities and the cytostatic activities of the test compounds, camostat mesilate did not reduce cell viability, nor did it inhibit MDCK cell proliferation at concentrations of up to 1 mg/ml. Thus, its SI was more than 500. The anti-influenza virus activity of camostat mesilate was confirmed in virus-infected chicken embryos.

The four protease inhibitors examined in this study inhibited the replication of influenza viruses A and B but not the replication of RSV, measles virus, or parainfluenza virus type 3 in vitro. They reduced the viral HA titer of influenza viruses A and B and parainfluenza virus type 1 in ovo. We compared the amino acid sequences of the putative cleavage-activation sites of the orthomyxovirus and paramyxovirus glycoproteins (Fig. 3) which are responsible for the virus-cell fusion process. Influenza viruses A (20) and B (1) and parainfluenza virus type 1 (2) have a single basic amino acid, whereas RSV (19), measles virus (18), and parainfluenza virus type 3 (18) have consecutive basic residues. Gotoh et al. (5) have recently characterized one of the virus-activating proteases, designated type 1 (VAP-1), from the allantoic fluid of embryonated chicken eggs. This protease activates the fusion glycoproteins of influenza virus A and parainfluenza virus type 1 by cleaving precursor proteins at a specific single arginine site. On the other hand, the fusion glycoprotein precursor of virulent Newcastle disease virus strains is intracellularly cleaved by virus-activating protease type 2 (VAP-2) at consecutive basic residue sites (14). These observations may suggest that protease inhibitors effectively inhibit VAP-1 activity but not VAP-2 activity, and they may thus explain why virus replication is inhibited by the protease inhibitors with some viruses (i.e., influenza viruses A and B and parainfluenza virus type 1), whereas with other viruses (i.e., RSV, measles virus, and parainfluenza virus type 3), virus replication is not affected, although the mechanisms of antiviral action of the protease inhibitors have not been resolved yet.

Camostat mesilate, which showed the greatest promise as a candidate drug for the treatment of influenza virus infection, is widely used in the treatment of pancreatitis. The concentration of camostat mesilate achievable in serum by oral administration of a usual dosage is 84 ng/ml. This concentration is significantly lower than that at which camostat mesilate inhibited the replication of influenza viruses in vitro and in ovo. It would be interesting to determine whether the effective concentration of the protease inhibitor is attainable in vivo.

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