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Inhibitory effects of aprotinin on influenza A and B viruses in vitro and in vivo

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Abstract

Background: Long-term or frequent use of currently approved anti-influenza agents has resulted in the emergence of drug-resistant viruses, necessitating the discovery of new drugs. In this study, we found aprotinin, a serine protease inhibitor, as an anti-influenza candidate through screening of compound libraries. Aprotinin has been previously reported to show inhibitory effects on a few subtypes (e.g., seasonal H1N1 and H3N2) of influenza A virus (IAV). However, there were no reports of its inhibitory effects on the other types of influenza virus. Therefore, in this study, we investigated the inhibitory effects of aprotinin against a wide range of influenza viruses in vitro and in vivo. Methods: We tested the antiviral activity of aprotinin in Madine-Darby canine kidney (MDCK) cells against seasonal human IAVs, avian influenza viruses with zoonotic potential, oseltamivir-resistant IAVs, and influenza B virus. We also tested the antiviral activity of aprotinin against A/PR/8/34 (H1N1) virus in a mouse model. Results: Our cell-based assay showed that aprotinin had inhibitory effects on seasonal human IAVs (H1N1 and H3N2 subtypes), avian IAVs (H5N2, H6N5, and H9N2 subtypes), an oseltamivir-resistant IAV, and a currently circulating influenza B virus. We have also confirmed its activity in mice infected with a lethal dose of influenza virus, showing a significant increase in survival rate. Conclusions: Our findings suggest that aprotinin has the capacity to inhibit a wide range of influenza virus subtypes and should be considered for development as a therapeutic agent against influenza.

1 INTRODUCTION

Influenza viruses remain important pathogens that cause respiratory diseases in humans and animals. Human seasonal influenza A and B viruses annually cause severe morbidity and economic losses worldwide. The Centers for Disease Control (CDC) estimates around 23,000 flu-related deaths in the United States each year.[1] In addition, avian influenza viruses, such as the H5 and H7 subtypes, sporadically cause highly lethal infections in both animals and humans,[2-4] and animal or human-animal influenza reassortant viruses occasionally cause global epidemic or pandemic influenza.[5]

Vaccination is considered the most effective strategy for controlling influenza in humans.[6] However, current influenza vaccines have several limitations, including their limited efficacy due to antigenic mismatches between the vaccine and circulating virus strains.[7] For this reason, antiviral drugs are important for controlling influenza. Representative classes of anti-influenza drugs include adamantane-based matrix protein 2 (M2) ion channel blockers (e.g., amantadine and rimantadine) and neuraminidase (NA) inhibitors (e.g., oseltamivir and zanamivir).[8] However, the emergence of antiviral drug resistance is a constant concern owing to the high mutation rates of influenza viruses.[9] Since the first report of amantadine-resistant influenza A viruses (IAVs) during the 1980 epidemic,[10] the prevalence of these viruses among circulating IAVs (especially, H1N1 and H3N2 subtypes) has increased rapidly to nearly 100% of the cases.[11] In response,

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the CDC has stopped recommending the use of adamantane in the United States.[12] Increasing application of NA inhibitors (especially oseltamivir) brings into focus the risk of developing resistance to this class of anti-influenza drugs. Although the prevalence of NA inhibitor-resistant influenza viruses is generally low (oseltamivir <3.5%) or rare (zanamivir <1%), [13-16] the problem of reduced susceptibility and resistance of influenza viruses to NA inhibitors has been increasing recently. Therefore, there is an utmost need to develop better or novel anti-influenza drugs.

In this study, we first aimed to identify anti-influenza viral agents by screening compound libraries. Aprotinin, a serine protease inhibitor, presented as a candidate. Previous reports have suggested that aprotinin has anti-influenza viral activity.[17-19] However, most reports cover only a narrow range of IAV strains (especially seasonal IAV strains) and strains of influenza B virus (IBV) that may no longer be circulating. Therefore, we investigated the anti-influenza viral effects of aprotinin on various subtypes of IAV, including i) human seasonal IAVs, ii) avian influenza viruses with zoonotic potential (H5N2, H9N2, and H6N5), iii) oseltamivir-resistant IAV, and iv) a currently circulating strain of IBV *in vitro* . We also used a mouse model to verify the anti-influenza activity of aprotinin. Our findings contribute further evidence to the potential of aprotinin as a broad-spectrum anti-influenza agent.

2 METHODS

2.1 Cells, viruses, and aprotinin

Madin-Darby canine kidney (MDCK) cells were cultured in Growth Medium: 1× Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), 3% L-glutamine (Gibco), 0.75% sodium bicarbonate (Gibco), 1% MEM vitamin solution (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml gentamicin (Gibco), and 1% antibiotic-antimycotic antibiotics solution (Gibco).

Seven IAVs and one IBV were used in this study: A/Puerto Rico/8/1934 (A/PR/8/34; H1N1); A/California/04/2009 (A/CA/04/09; H1N1); A/Philippines/2/1982 (A/PH/2/82; H3N2); A/Brisbane/10/2007 (A/Bris/10/07; H3N2); A/Aquatic Bird/Korea/CN2/2009 (A/AB/Kor/CN2/09; H5N2); A/Aquatic Bird/Korea/CN5/2009 (A/AB/Kor/CN5/09; H6N5); A/Chicken/Korea/01310/2001 (A/Ck/Kor/01310/01; H9N2); and B/Seoul/32/2011 (B/Seoul/32/11). The IAVs were grown in 10-day-old embryonated chicken eggs for 48 h at 35 °C. The allantoic fluid was harvested, and aliquots were stored at -70 degC until use. The IBV was propagated in MDCK cells in Infection Medium: 1x MEM supplemented with 0.3% bovine serum albumin (Sigma-Aldrich) (instead of FBS) and 1.0 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA). After incubation for 72 h, the supernatant was harvested, and aliquots were stored at -70 °C until use.

Aprotinin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was dissolved in distilled water to make stock solutions, and aliquots were stored at -20 degC until use.

2.2 Cell viability and cytotoxicity assays

For the cell viability assay, MDCK cells were cultured on a 96-well plate (2 x 10^4 cells/well) in Growth Medium. After incubation for 16 h at 37 degC, the cells were infected with each influenza virus at 10^3 50% tissue culture infectious dose ($TCID_{50}$)/well and were washed with phosphate-buffered saline (PBS) at 1 h post-infection. Various concentrations (10 to 200 nM, n = 3 per dose) of aprotinin diluted with Infection Medium were added into each well to a final volume of 100 μl/well. After incubation for 72 h at 37 °C, cell viability was determined using the EZ-Cytox kit (Daeillab service Co., South Korea) according to the manufacturer's instructions. Cell viability was indicated by percentage values, compared to the negative control (cells that were infected but not treated with aprotinin). Cytotoxicity of aprotinin was measured similarly as described above, but without infecting with the virus. Cytotoxicity was presented as a percentage value, compared to the negative control (wells containing cells only).

2.3 Virus growth kinetics

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MDCK cells were cultured on a 24-well plate (1.25×10^5 cells/well) in Growth Medium. After incubation for 16 h at 37 °C, the cells were infected with each influenza virus at a multiplicity of infection (MOI) of 0.01 or 0.001 and were washed with PBS at 1 h post-infection. The cells were treated with aprotinin (60 nM/well) or oseltamivir (100 μM/well; positive control) in a total volume of 0.5 ml Infection Medium/well. The culture supernatant was collected at 0, 16, 24, 32, 48, and 64 h post-infection and stored at -70 °C until analysis.

Virus titration was performed using MDCK cells. The cells were cultured on a 96-well plate (2×10^4 cells/well) in Growth Medium and were infected with 100 μl of serial tenfold dilutions of the culture supernatant in Infection Medium. After incubation for 72 h at 37 °C, the culture supernatant was harvested to determine virus titration by the hemagglutination assay using chicken red blood cells. The virus titers were determined by calculating the $TCID_{50}$ using the Reed-Muench method.[20]

2.4 Mouse experiments

All animal experiments were conducted in biosafety level 2-plus facilities at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea). General animal care was provided as required by the Institutional Animal Care and Use committee. Six- to eight-week-old C57BL/6 mice were purchased from Koatech (Pyeongtaek, South Korea), and ten mice per group were used. The mice were inoculated via intranasal instillation with 3 times the 50% lethal dose (3 LD_{50}) of the PR8 virus. The following day, the mice were treated intravenously with aprotinin (2 mg/kg/day), orally with oseltamivir (10 mg/kg/day), or intravenously with PBS (negative control) twice a day for 5 days. The mice were monitored daily for 14 days for weight change and mortality.

2.5 Data analysis

Based on the inhibition of virus growth, the half-maximal effective concentration (EC_{50}) of aprotinin was calculated using the GraphPad Prism program (version 5; San Diego, CA, USA). This procedure is commonly known as a logistic regression using the following formula: $Y = 1/1 + 10$ ($[\log EC_{50} - \log X] \times$ Hillslope), where Y represents response (inhibition of virus growth), and X represents the concentration of aprotinin. Hillslope is the parameter to describe the steepness of the curve. The data analysis was performed using GraphPad Prism for Windows, and differences were evaluated by one-way ANOVA. P values less than 0.05 ($P< 0.05$) were considered statistically significant.

3 RESULTS

3.1 Screening of compound libraries for inhibitors of influenza A virus

To develop better or novel antiviral drugs against influenza virus infection, we screened compound libraries through the MDCK cell-based screening system using A/PR/8/34 (H1N1) virus, the standard strain of human IAV. We tested a total of 1,280 compounds and found 13 anti-influenza candidates. Aprotinin, a serine protease inhibitor, was selected as a final candidate because both aprotinin samples from this library and from that of another company showed inhibitory effects on the virus (data not shown). Virus inhibition assay in MDCK cells showed that aprotinin inhibits A/PR/8/34 (H1N1) in a dose-dependent manner (Fig. 1A). Aprotinin could also inhibit A/CA/04/09 (H1N1), A/PH/2/82 (H3N2), A/AB/Kor/CN05/09 (H6N5), A/Ck/Kor/01310/01 (H9N2), A/Bris/10/07 (H3N2), and B/Seoul/32/11 in a dose-dependent manner (Fig. 1B-G). Based on the results of the viral inhibition assay, we applied the lowest effective concentration against all the tested influenza strains (60 nM) for subsequent experiments.

We next compared the antiviral activity of aprotinin against A/PR/8/34 (H1N1) virus with that of oseltamivir (100 μM). Aprotinin showed corresponding or superior antiviral activity to oseltamivir against PR8 virus infection (Fig. 2A). Colorimetric cytotoxicity assay revealed that there was no cytotoxicity in the range of aprotinin concentrations tested in this study ([?]200 nM) (Fig. 2B).

3.2 *In vitro* inhibitory effects of aprotinin on infections caused by various influenza A virus subtypes

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Previous studies have minimal information on the spectrum of the anti-influenza viral activity of aprotinin. Therefore, we evaluated the effects of aprotinin treatment on the production of infectious particles of various IAV subtypes, including human and avian viruses, through time-based studies by determining the growth kinetics of the following viruses in MDCK cells: A/PR/8/34 (H1N1), A/CA/04/09 (H1N1), A/PH/2/82 (H3N2), A/AB/Kor/CN2/09 (H5N2), A/AB/Kor/CN5/09 (H6N5), and A/Ck/Kor/01310/01 (H9N2) viruses. We simultaneously added each virus and 60 nM aprotinin to the cell cultures and cultured without removing either virus or aprotinin throughout the incubation period. Culture supernatants were collected at different time points, and virus titers were determined by calculating the median $TCID_{50}$ based on the hemagglutination assay. As shown in Fig. 3, aprotinin was able to significantly reduce the production of the tested human IAVs after more than 16 h post-infection, supporting the results of previous studies.[17,19] Especially, aprotinin had superior inhibitory effects on A/CA/04/09 (H1N1) and A/PH/2/82 (H3N2) viruses than oseltamivir. Aprotinin was also able to significantly reduce the production of the tested avian IAVs (Fig. 4). Aprotinin displayed weaker inhibitory effects on avian IAVs than oseltamivir at early time points post-infection but presented similar effects to oseltamivir at 64 h post-infection. These results indicate that aprotinin had inhibitory effects on infections of both human and avian IAVs.

3.3 *In vitro* inhibitory effects of aprotinin on infections caused by oseltamivir-resistant influenza A and B viruses

We next examined the inhibitory effects of aprotinin against an oseltamivir-resistant IAV (A/Bris/10/07; H3N2). As shown in Fig. 4A, A/Bris/10/07 (H3N2) virus was less susceptible to oseltamivir. Our genetic analysis revealed that the H28T mutation in HA conferred the reduced susceptibility of the H3N2 virus to oseltamivir (data not shown). However, aprotinin could effectively reduce the production of the H3N2 virus (Fig. 5A).

IBVs are generally less susceptible to oseltamivir than IAVs.[21,22] Therefore, we here investigated the inhibitory effects of aprotinin on influenza B virus (B/Seoul/32/2011) infection. As shown in Fig. 5B, the IBV was around 50- to 100-fold less susceptible to oseltamivir. However, aprotinin was more effective than oseltamivir at reducing IBV production.

Taken together, these results suggest that aprotinin can significantly reduce the production of oseltamivir-resistant IAV and of IBV, which is less susceptible to oseltamivir.

3.4 Determining the EC_{50} values of aprotinin for various influenza A and B viruses

To further characterize the effects of aprotinin on various subtypes of influenza A and B viruses, we determined its EC_{50} values in MDCK cells against different influenza subtypes. We treated MDCK cells infected with different influenza virus strains with varying concentrations (10-200 nM) of aprotinin (Fig. 6). Aprotinin inhibited the infection of several influenza viral strains in a dose-dependent manner. The calculated EC_{50} value for aprotinin against each influenza strain is shown in Table 1.

3.5 Inhibitory effects of aprotinin treatment in mice infected with a lethal dose of A/PR/8/34 (H1N1) virus

To test whether aprotinin has antiviral activity *in vivo* , we tested its effects against lethal A/PR/8/34 (H1N1) virus infection in C57BL/6 mice. We initially treated mice with once-daily intravenous injections of aprotinin. However, while it did not have toxic effects*in vivo* , it also did not display antiviral effects in influenza-infected mice (data not shown). The initial half-life of aprotinin may be too short for conferring antiviral effects *in vivo* . As such, we decided to administer aprotinin twice a day.

C57BL/6 mice were intranasally inoculated with 3 $LD_{50}$of A/PR/8/34 (H1N1) virus. For 5 days after infection, the mice received twice-daily intravenous injections of aprotinin or twice-daily oral administrations of oseltamivir or PBS as controls. Bodyweight changes and survival were monitored daily for 14 days following infection (Fig. 7). The PBS-treated mice had 0% survival at 8 days post-infection. Meanwhile, the groups of mice treated with either aprotinin or oseltamivir showed 75% or 100% survival. These results strongly support the antiviral effect of aprotinin against influenza virus infection.

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4 DISCUSSION

Given the limitations of influenza vaccines and the recent rise in the number of oseltamivir-resistant strains, there remains a need to discover and develop new anti-influenza agents. In our cell culture-based screening of compound libraries, aprotinin was identified as a strong anti-influenza candidate. It has been previously reported as an anti-influenza agent *in vitro* ,[23] in embryonated chicken eggs,[18,24] and in mice.[25] It is currently licensed in Russia for clinical use in aerosolized form (Aerus), primarily against seasonal H1N1 and H3N2 influenza, but it has also been tested against H2N2 and avian-like H7N9 influenza viruses.[17,19] Aprotinin is a naturally occurring non-specific inhibitor of serine proteases, including trypsin, chymotrypsin, plasmin, and kallikrein.[26] Influenza viruses require proteolytic cleavage and structural rearrangement of hemagglutinin (HA) for successful fusion with host endosomes. The HA precursor protein, HA0, is cleaved into HA1 and HA2, which are initially linked by a short peptide sequence. Trypsin-like proteases facilitate this cleavage by targeting arginine in the linker peptide of most influenza virus strains. Aprotinin is believed to inhibit HA0 cleavage by competing for the active site of these proteases.

Since previous studies have shown that aprotinin inhibited a limited number of subtypes of IAV (mainly H1N1 and H3N2), we decided to examine its antiviral activity against a broader range of influenza virus in this study. The tested strains included avian strains of IAV, an oseltamivir-resistant strain of IAV, and a strain of IBV.

Similar to previous reports, we found that aprotinin was able to inhibit the production of seasonal H1N1 and H3N2 IAVs in MDCK cells. The effects of aprotinin were either comparable or superior to the effects of oseltamivir. We also found that aprotinin could inhibit avian IAVs belonging to the H9N2, H5N2, and H6N5 subtypes *in vitro* at levels similar or superior to those of oseltamivir. H9N2 currently circulates in poultry and is generally avirulent or low-pathogenic. However, occasional outbreaks in poultry farms have occurred, and sporadic human infection cases have also been reported.[27] Both the H5N2 and H6N5 viruses in this study were isolated from wildfowl in South Korea. The H6N5 isolate was found to cause considerable morbidity and mortality in mice without bearing any known pathogenicity marker.[28] Meanwhile, the H5N2 isolate, adapted to and caused lethality in mice after only a single lung-to-lung passage.[29] Evidently, these isolates have the capacity to easily cross the avian-mammalian transmission barrier and may emerge as zoonotic agents in the future. The ability of aprotinin to inhibit these avian influenza viruses suggests that aprotinin may potentially be used in human outbreaks of avian influenza viruses.

We have also shown that aprotinin is able to inhibit an oseltamivir-resistant influenza A strain (A/Bris/10/07; H3N2). Additionally, similar to earlier reports of aprotinin's activity against the B/Lee/40 and B/HK/73 viruses,[18] aprotinin shows antiviral activity against a currently circulating strain of IBV (Yamagata-like lineage, B/Seoul/32/2011). IBVs are generally less susceptible to oseltamivir, especially in children.[30,21] Because aprotinin targets a host factor required for infection, influenza viruses are less likely to develop aprotinin resistance, especially since trypsin-like proteases, the targets of aprotinin, are required for influenza virus proliferation. Therefore, the use of aprotinin may be more beneficial in the long run than the use of drugs targeted against viral components.

Aprotinin was commonly indicated as a prophylactic agent to prevent blood loss and to reduce the need for blood transfusions in cardiac bypass surgeries. However, due to safety concerns, aprotinin had been pulled out of the market in 2007; it has since been re-licensed in Canada and Europe for the same application.[31] It was generally well-tolerated in animal models and in clinical trials, and it is given at high intravenous doses for human application, suggesting that it is safe to use at high doses.[32] In our study, at least twice-daily intravenous administrations were needed for aprotinin to be protective against influenza virus infection in a mouse model. Aprotinin has a relatively short plasma half-life (0.7–2 h), and 90% of the administered dose is absorbed by the kidney in a few hours,[33] which requires aprotinin to be applied in high-dose intravenous administrations in surgeries.[32] This probably explains why once-daily intravenous administrations were not sufficient to exert inhibitory effects against the influenza virus. As such, high plasma concentrations of aprotinin may also be required to inhibit influenza viruses. However, as in the case of the licensed aerosolized aprotinin in Russia, multiple doses of intranasally administered aprotinin

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may be more beneficial for application against influenza virus infection in humans.[25,34,17,19] This way, aprotinin does not have to circulate systemically and will be targeted in the upper respiratory tract, where most influenza virus subtypes replicate in humans. However, in this study, we did not test the intranasal administration of aprotinin. Future studies will have to be performed to determine the optimal dosage and route of administration for human application. Additionally, whether aprotinin will be effective against highly pathogenic avian influenza viruses (HPAIVs) will have to be evaluated. HPAIVs have multibasic cleavage sites that are more accessible to a wide range of proteases.[35] If aprotinin has the ability to inhibit HPAIVs, then it will be a viable pandemic influenza therapeutic candidate that runs a lower risk of causing drug resistance than currently used antivirals like oseltamivir.

In this study, we were able to demonstrate that aprotinin inhibits*in vitro* production of 1) avian IAVs with zoonotic potential, 2) oseltamivir-resistant IAV, and 3) currently circulating IBV, which is inherently less susceptible to oseltamivir. We propose that aprotinin is an excellent candidate for the treatment of most IAVs and IBVs in humans. However, whether aprotinin is similarly effective against HPAIVs together with the selection of the most appropriate route of administration and the optimal dosage for its clinical use still needs to be determined in future studies.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Eun-Jung Song: Data curation (equal); Formal analysis (equal); Investigation (equal); Software (lead); Validation (equal); Visualization (equal); Writing-original draft (lead). **Erica Español:** Data curation (equal); Formal analysis (equal); Investigation (equal); Validation (equal); Visualization (equal); Writing-review & editing (lead). **Sang-Mu Shim:** Data curation (equal); Formal analysis (equal); Investigation (equal); Validation (equal); Visualization (equal). **Jeong-Hyun Nam:** Investigation (supporting); Validation (supporting); Visualization (supporting).**Jiyeon Kim:** Investigation (supporting); Validation (supporting); Visualization (supporting). **Kiho Lee:**Investigation (supporting); Validation (supporting); Visualization (supporting). **Chong-Kil Lee:** Conceptualization (equal), Methodology (supporting), Resources (lead), Supervision (supporting), Writing-review & editing (supporting). **Jeong-Ki Kim:**Conceptualization (equal), Data curation (equal); Formal analysis (equal); Investigation (equal); Validation (equal); Visualization (equal); Writing-original draft (lead), Writing-review & editing (lead).

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Table 1. Half-maximal effective concentrations (EC_{50}) of aprotinin against various influenza virus strains in MDCK cells

Type	Virus	EC_{50}
A	A/PR/8/34 (H1N1)	14 nM
A	A/CA/04/09 (H1N1, 2009 pandemic)	11 nM
A	A/PH/2/82 (H3N2)	21 nM
A	A/AB/Kor/CN5/09 (H6N5)	87 nM
A	A/Ck/Kor/01310/01 (H9N2)	57 nM
A	A/Bris/10/07 (H3N2, oseltamivir-resistant)	110 nM
B	B/Seoul/32/11 (Yamagata-like)	39 nM

Figure Legends

Figure 1 Dose-dependent effects of aprotinin against influenza viruses *in vitro* . Madine-Darby canine kidney (MDCK) cells were infected with 100 or 1000 $TCID_{50}$/ml of (A) A/PR/8/34 (H1N1), (B) A/CA/04/09 (H1N1), (C) A/PH/2/82 (H3N2), (D) A/AB/Kor/CN5/09 (H6N5), (E) A/Ck/Kor/01310/01 (H9N2), (F) A/Bris/10/07 (H3N2), or (G) B/Seoul/32/11 (Yamagata-like lineage) and were treated with varying concentrations of aprotinin (10-200 nM; n = 3 per dose) for 72 h. Cell viability was measured using the EZ-Cytox reagent, and percent virus inhibition was calculated relative to the uninfected MDCK cell viability (cell-only control). $TCID_{50}$: median tissue culture infectious dose.

Figure 2 Antiviral effect of aprotinin compared with oseltamivir and cytotoxicity assay. (A) Madine-Darby canine kidney (MDCK) cells were infected with A/PR/8/34 (1000 $TCID_{50}$/ml) and treated with 60 nM aprotinin or 100 μM oseltamivir. Untreated and uninfected MDCK cells (cell) and untreated infected cells (PR8) were used as controls. (B) To determine the cytotoxicity of aprotinin, the cell viability was measured by treating the MDCK cells with the compound for 72 h and compared with that of untreated control cells. Cell viability was measured using EZ-Cytox. The experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, statistically significant difference between the PR8 and PR8 + oseltamivir or PR8 + aprotinin groups. $TCID_{50}$: median tissue culture infectious dose.

Figure 3 Aprotinin inhibited the replication of various strains of human influenza A virus in MDCK cells. The replication kinetics of (A) A/PR/8/34 (H1N1), (B) A/CA/04/09 (H1N1), and (C) A/PH/2/82 (H3N2) virus were investigated in Madine-Darby canine kidney (MDCK) cells after treatment with aprotinin and oseltamivir. MDCK cells were infected with influenza virus at an MOI of 0.01 in the presence of aprotinin (60 nM) or oseltamivir (100 μM), and supernatants were harvested at 0, 16, 24, 32, 48, and 64 h. The

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virus titer in the supernatants was determined by $TCID_{50}$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, statistically significant difference between the virus-only (media) group and the aprotinin treatment group. MOI: multiplicity of infection; $TCID_{50}$: half-maximal tissue culture infectious dose.

Figure 4 Aprotinin inhibited the replication of various strains of avian influenza A virus in MDCK cells. The replication kinetics of (A) A/AB/Kor/CN2/09 (H5N2), (B) A/AB/Kor/CN5/09 (H6N5), and (C) A/Ck/Kor/01310/01 (H9N2) were investigated in Madine-Darby canine kidney (MDCK) cells after treatment with aprotinin and oseltamivir. MDCK cells were infected with influenza virus at an MOI of 0.01 (H5N2 and H9N2) or 0.001 (H6N5) in the presence of aprotinin (60 nM) or oseltamivir (100 μM), and supernatants were harvested at 0, 16, 24, 32, 48, and 64 h. The virus titer in the supernatants was determined by $TCID_{50}$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, statistically significant difference between the virus-only (media) group and the aprotinin treatment group. MOI: multiplicity of infection; $TCID_{50}$: half-maximal tissue culture infectious dose.

Figure 5 Aprotinin inhibited the replication of oseltamivir-resistant influenza viruses in MDCK cells. The replication kinetics of (A) A/Bris/10/07 (H3N2) and (B) B/Seoul/32/11 were investigated in Madine-Darby canine kidney (MDCK) cells after treatment with aprotinin and oseltamivir. MDCK cells were infected with influenza virus at an MOI of 0.01 (H3N2) or 0.001 (B/Seoul/32/11) in the presence of aprotinin (60 nM) or oseltamivir (100 μM), and supernatants were harvested at 0, 16, 24, 32, 48, and 64 h. The virus titer in the supernatants was determined by $TCID_{50}$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, statistically significant difference between the virus-only (media) group and the aprotinin treatment group. MOI: multiplicity of infection; $TCID_{50}$: half-maximal tissue culture infectious dose.

Figure 6 Concentration-dependent reduction in the replication of various influenza virus strains in MDCK cells following treatment with aprotinin. To calculate the half-maximal effective concentrations (EC_{50}) of aprotinin against the different influenza virus strains, Madine-Darby canine kidney (MDCK) cells were infected with (A) A/PR/8/34 (H1N1, 1000 $TCID_{50}$/ml), (B) A/CA/04/09 (H1N1, 1000 $TCID_{50}$/ml), (C) A/PH/2/82 (H3N2, 1000 $TCID_{50}$/ml), (D) A/AB/Kor/CN5/09 (H6N5, 100 $TCID_{50}$/ml), (E) A/Ck/Kor/01310/01 (H9N2, 1000 $TCID_{50}$/ml), (F) A/Bris/10/07 (H3N2, 1000 $TCID_{50}$/ml), and (G) B/Seoul/32/11 (100 $TCID_{50}$/ml) treated with varying doses of aprotinin (10-200 nM). Three days after virus infection, cell viability was measured using EZ-Cytox, and each data point represents the average of the experiment performed in triplicate cell cultures. $TCID_{50}$: half-maximal tissue culture infectious dose.

Figure 7 Antiviral effect of aprotinin against influenza A virus in mice. Groups of mice (n = 8 per group) were intranasally infected with A/PR/8/34 (H1N1) virus at three times the 50% mouse lethal dose (3 LD_{50}). Oseltamivir was orally administered twice a day at 10 mg/kg/day, and aprotinin was intravenously administered at 2 mg/kg/day, twice a day for 5 days. (A) Change in body weight of mice and (B) survival rates were monitored for 2 weeks. $^{***}P < 0.001$, a statistically significant difference between the negative control group (virus-infected only) and the aprotinin treatment group.

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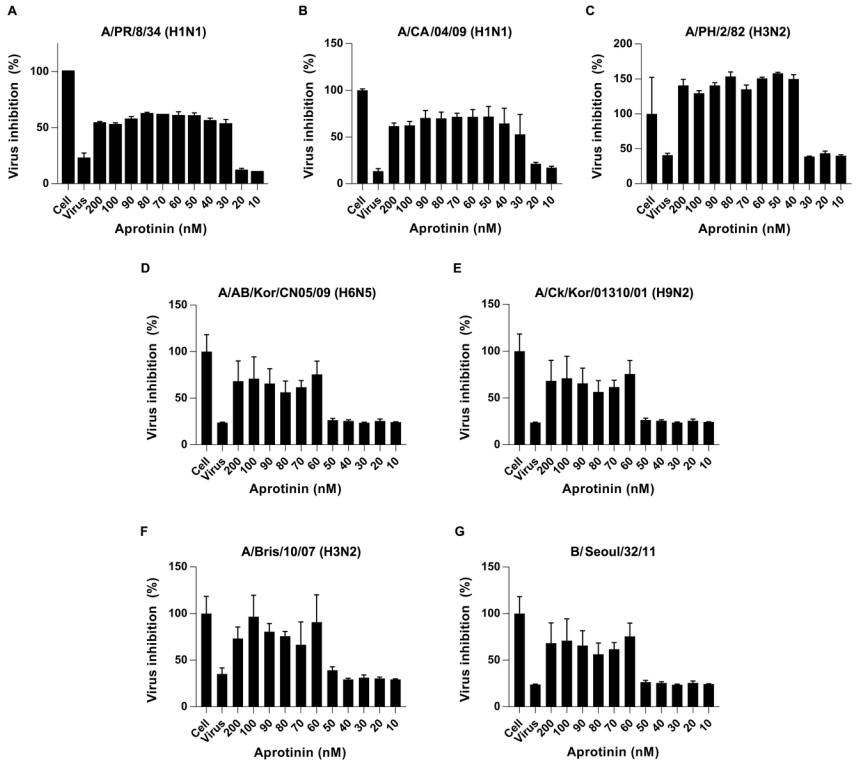

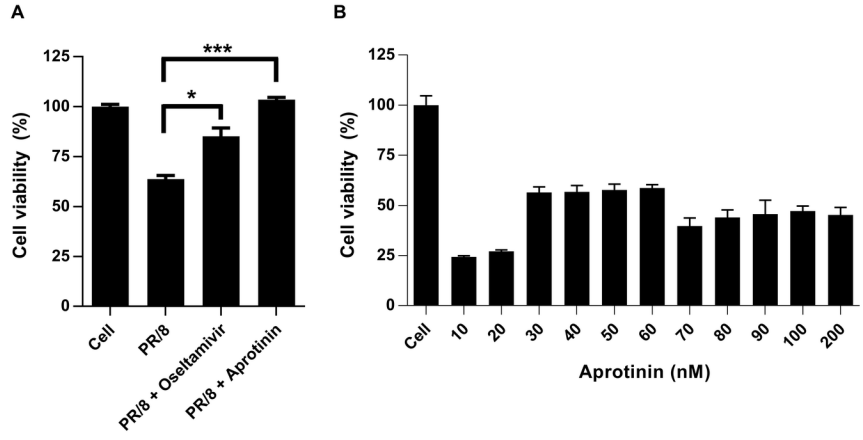

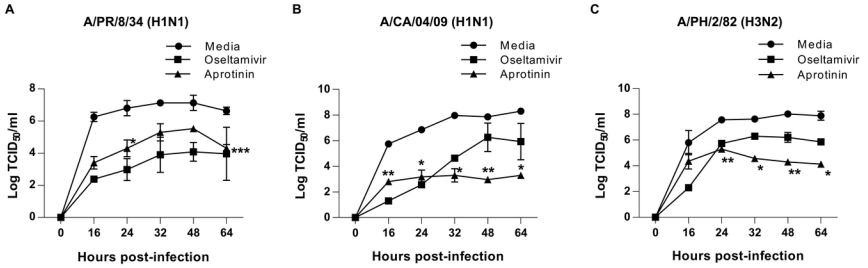

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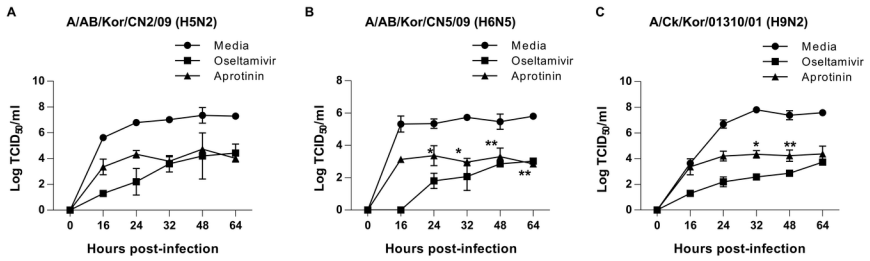

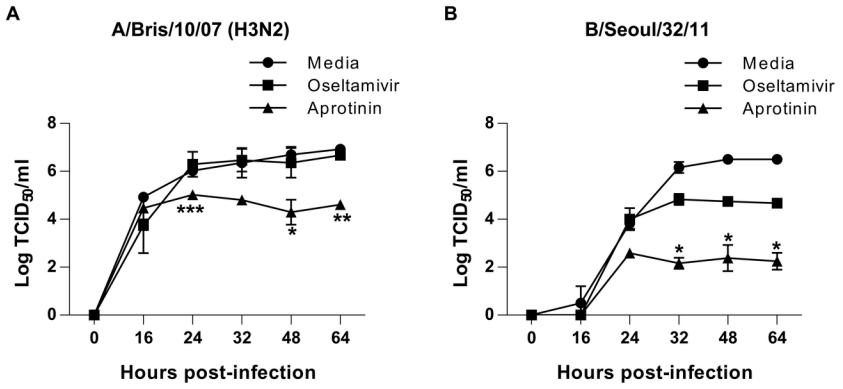

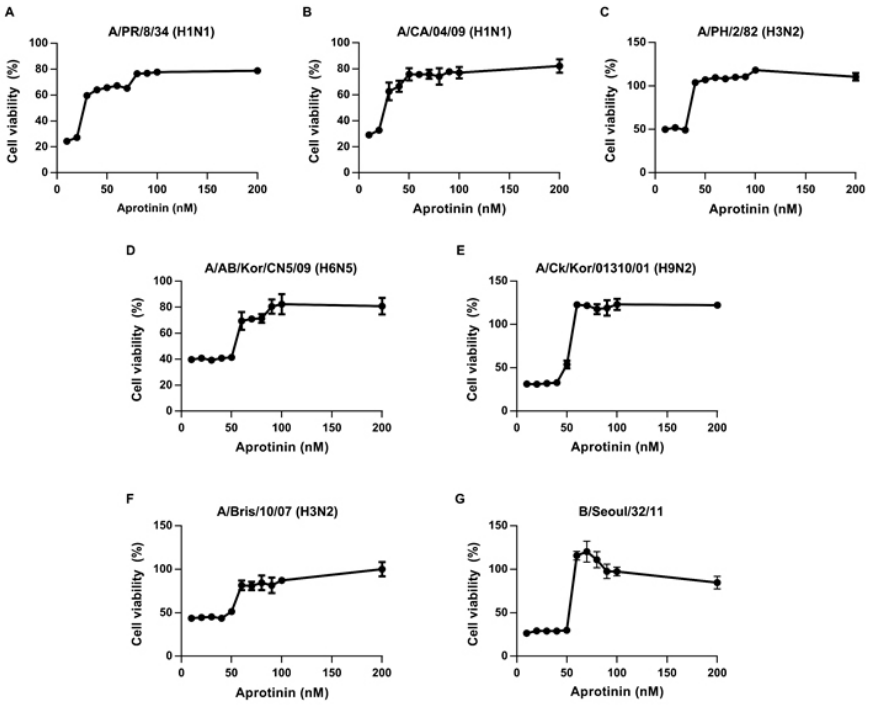

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