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Synergistic antiviral effect of *Galanthus nivalis* agglutinin and nelfinavir against feline coronavirus

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ABSTRACT

Feline infectious peritonitis (FIP) is a fatal disease in domestic and nondomestic felids caused by feline coronavirus (FCoV). Currently, no effective vaccine is available for the prevention of this disease. In searching for agents that may prove clinically effective against FCoV infection, 16 compounds were screened for their antiviral activity against a local FCoV strain in *Felis catus* whole fetus-4 cells. The results showed that *Galanthus nivalis* agglutinin (GNA) and nelfinavir effectively inhibited FCoV replication. When the amount of virus preinoculated into the test cells was increased to mimic the high viral load present in the target cells of FIP cats, GNA and nelfinavir by themselves lost their inhibitory effect. However, when the two agents were added together to FCoV-infected cells, a synergistic antiviral effect defined by complete blockage of viral replication was observed. These results suggest that the combined use of GNA and nelfinavir has therapeutic potential in the prophylaxis and treatment of cats with early-diagnosed FIP.

1. Introduction

Feline infectious peritonitis (FIP) is a fatal disease in domestic and nondomestic felids caused by feline coronavirus (FCoV) (Hartmann, 2005). This immunopathogenic disease of cats is similar to severe acute respiratory syndrome (SARS) in humans; both are characterized by an intense inflammatory response that compromises normal physiological function and contributes to a progressive debilitating condition, weight loss, fever, and systemic disease (Paltrinieri, 2004; Perlman and Dandekar, 2005).

Although FCoV has been known for more than 40 years to be the causative pathogen of FIP, the immunopathogenic nature of the disease has prevented the success of vaccination trials (Christianson et al., 1989; Scott, 1987; Stoddart et al., 1988; Vennema et al., 1990a,b). Several studies have attempted to identify effective anti-FCoV treatments for FIP-diseased cats (Hartmann and Ritz, 2008). Ribavirin, a nucleoside analogue, was shown to inhibit the growth of FCoV in vitro (Barlough and Scott, 1990; Weiss and Oostrom-Ram, 1989); nevertheless, the side effects hindered its clinical application (Weiss et al., 1993).

Recently, several antiviral agents against SARS-coronavirus (SARS-CoV) were identified. These included carbohydrate-binding agents (Keyaerts et al., 2007), HIV protease inhibitors (Yamamoto et al., 2004), an antipsychotic drug (Ho et al., 2007; Zhang and Yap, 2004), an anthraquinone compound (Ho et al., 2007), a nucleoside analogue (Tan et al., 2004), and an interferon subtype. Because some of these SARS-CoV inhibitors and other commercially available antiviral agents might be promising candidates for controlling and treating FCoV infection in cats, we carried out the present study and successfully identified two compounds that act effectively against a recently isolated FCoV strain. We found that the combined use of these agents shows a synergistic antiviral effect.

2. Materials and methods

2.1. Test compounds

In this study, 16 compounds were used. Based on pharmacological activity, these antiviral agents were grouped as (i) nucleoside analogues: acyclovir, idoxuridine, and ribavirin; (ii) protease inhibitors: atazanavir, indinavir, lopinavir/ritonavir, nelfinavir, and saquinavir; (iii) reverse transcriptase inhibitors: efavirenz, lamivudine, lamivudine/zidovudine, nelvirapine, and stavudine; (iv) compounds with other activities: emodin, *Galanthus nivalis* agglutinin (Atrasheuskaya et al., 2003), and promazine. Details of each compound tested are listed in Table 1. Besides indinavir and

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Table 1
Activity of compounds against FCoV in fcwf-4 cells.

Compound	Source	Highest concentration tested	CC_{50}	Inhibition of CPE formation	IC_{50}
Nucleoside analogues					
Acyclovir	GlaxoSmithKline (London, UK)	888 μM	≥888 μM	–	NA
Idoxuridine	Pharmacy compound	141.2 μM	≥141.2 μM	–	NA
Ribavirin	Roche (Basel, Switzerland)	12.8 μM	≥409.49 μM	–	NA
Protease inhibitors					
Atazanavir	Bristol-Myers Squibb (New York, USA)	17.75 μM	48.59 μM	–	NA
Indinavir	Merck (Whitehouse station, USA)	70.237 μM	≥70.237 μM	–	NA
Lopinavir/ritonavir	Abbott Laboratories (Abbott Park, USA)	3.125 μg/mL	47.04 μg/mL	–	NA
Nelfinavir	Roche (Basel, Switzerland)	9.41 μM	11.45 μM	+	8.19 μM
Saquinavir	Roche (Basel, Switzerland)	4.658 μM	95.83 μM	–	NA
Reverse transcriptase inhibitors					
Efavirenz	Merck (Whitehouse station, USA)	9.899 μM	52.14 μM	–	NA
Lamivudine	GlaxoSmithKline (London, UK)	27.257 μM	≥27.257 μM	–	NA
Lamivudine/zidovudine	GlaxoSmithKline (London, UK)	12.5 μg/mL	≥12.5 μg/mL	–	NA
Nelvirapine	Boehringer Ingelheim (Ingelheim am Rhein, Germany)	46.94 μM	≥46.94 μM	–	NA
Stavudine	Bristol-Myers Squibb (New York, USA)	55.754 μM	≥55.754 μM	–	NA
Other					
Emodin	Sigma, E7881 (St Louis, USA)	12.5 μM	67.41 μM	–	NA
Galanthus nivalis agglutinin	Sigma, L8275 (St Louis, USA)	0.48 nM	≥1.92 nM	+	0.0088 nM
Promazine	Sigma, P6656 (St Louis, USA)	100 nM	≥100 nM	–	NA

NA: not available.

GNA, which were dissolved in double distilled water, the solvent for all the other compounds was dimethyl sulfoxide.

2.2. Cells and virus

Felis catus whole fetus-4 (fcwf-4) cells (kindly provided by Professor Peter J. M. Rottier, Utrecht University) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin in 5% CO_2 at 37 °C. FCoV/NTU156/P/'07 (NTU156) is an FCoV strain recently isolated from pleural effusion of a kitten by the cocultivation method (Lin et al., 2009b). This local FCoV strain is a type II virus. The sequence of its partial spike gene was deposited in GenBank under the access number EU513388 (Lin et al., 2009a). All the viruses used in this study for the assessment of antiviral activity came from a stock passaged 12 times.

2.3. Cell viability

Cell viability was evaluated by the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 20,000 fcwf-4 cells per well were seeded in 96-well plates. After a 24 h incubation at 37 °C, various concentrations of compounds were added to confluent cell monolayers and incubated for 72 h. The highest concentration tested for each compound was indicated in Fig. 1. MTT was added to each well to a final concentration of 10 mg/mL. After 4 h of incubation at 37 °C, the medium containing MTT was removed, and the cells were lysed with 100 μL of lysis buffer. Following overnight incubation at room temperature, the absorbance value at 570 nm was measured at a wavelength of 655 nm using a microplate reader. Cell viability (%) was calculated from the expression: (OD of treated cells/OD of untreated cells) × 100. The concentration of the compound that reduced cell viability by 50% (CC_{50}) was determined using the MTT assay.

2.4. Screening of antiviral effects

Based on the results obtained from the cell viability tests, the highest concentration showing less than 5% cytotoxicity in fcwf-4 cells was chosen for each of the test compounds (Table 1); these chosen concentrations were premixed with NTU156 at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C for 1 h to monitor the effect on the preentry step. The mixtures of compound and virus were then inoculated into fcwf-4 cells in 24-well plates for 1 h of adsorption, and followed by the replacement of mixtures by DMEM with 2% FBS to monitor the effect on the entry step. To cope with the fast-growing nature of our NTU156 strain (Lin et al., 2009b), the infected cells were fixed at 15 h postinfection with 10% formalin and stained with 2% crystal violet. The cytopathic effect (CPE) was characterized by the formation of polykaryocytes. The number of polykaryocytes present in the whole well was counted under an inverted microscope.

2.5. Concentration-dependence of antiviral effects

The agents that were able to significantly inhibit the formation of CPE foci were further applied to evaluate antiviral effects in a concentration-dependent manner. Four concentrations showing <5% cytotoxicity of the effective compounds were chosen and premixed with NTU156 at MOI 0.01. After 1 h of incubation at 37 °C, the mixture was then inoculated into fcwf-4 cells in 24-well plates. Following 1 h of adsorption, the mixture was removed, and DMEM with 2% FBS was added. At 15 h postinfection, the cells were fixed and stained, and the CPE was counted.

2.6. Fifty percent of inhibitory concentration (IC_{50})

The inhibitory effect of the effective compounds was detected using a microtitration infectivity-inhibition assay. Each compound was twofold serially diluted and premixed with virus (MOI 0.01), and then inoculated into fcwf-4 cells and examined 72 h postinfection for the presence of viral CPE. The IC_{50} was calculated using the previously described method (Reed and Muench, 1938).

2.7. Combined use of the effective compounds in fcwf-4 cells

The fcwf-4 cells were infected with virus at MOI 0.1 for 1 h of adsorption and then the virus was removed and cells were maintained in fresh DMEM containing 2% FBS and effective compounds alone or in combination. At 48 h postinfection, the culture super-