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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.

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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
<i>Galanthus nivalis</i> 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% CO₂ 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-dimethylthiazol-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% (CC₅₀) 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 (IC₅₀)

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 IC₅₀ 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-

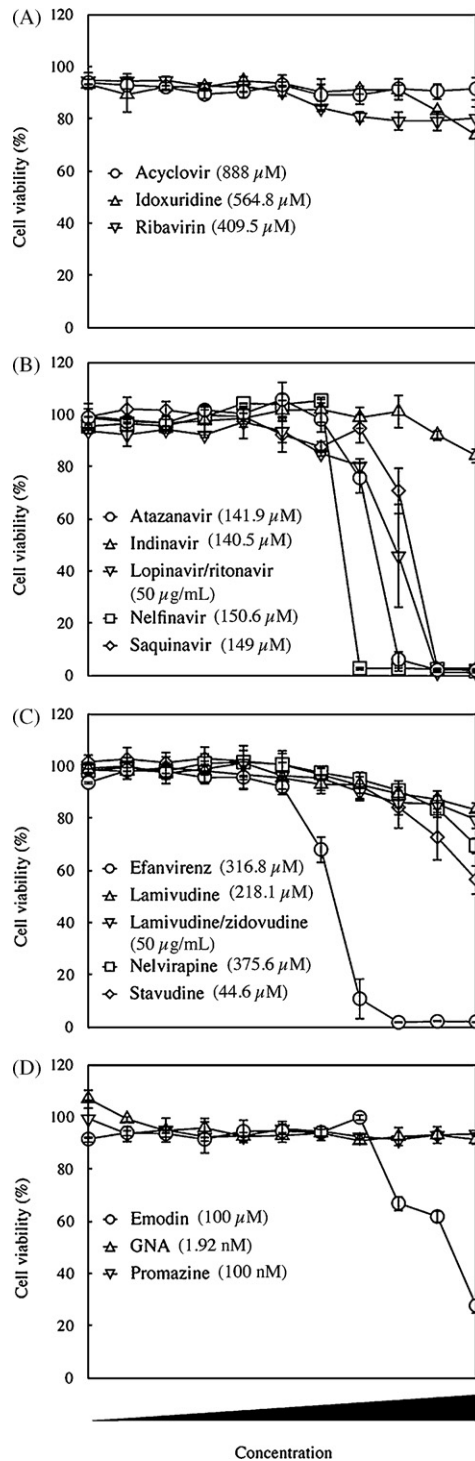


Fig. 1. The cytotoxic effects of each kind of antiviral compound in fcwf-4 cell. All the compounds were twofold serially diluted and added to the cells for 72 h of incubation. Cell viability was evaluated by MTT assay. (A) Nucleoside analogues. (B) Protease inhibitors. (C) Reverse transcriptase inhibitors. (D) Agents with other activities. The highest concentration tested of each compound was shown in parentheses. All compounds were analyzed in triplicate.

nantant was harvested and stored at -80°C before evaluation of the infectivity, and the cells were subjected to the MTT assay for assessing cell viability. The infectivity of the culture supernatants was evaluated by the plaque assay described by the method of Lin et al. (2009b). The viral inoculum was aspirated and overlaid with 1.5% methylcellulose DMEM supplemented with 2% FBS. After three days

of incubation, the cells were fixed and stained. The plaques were counted visually.

2.8. Statistical analysis

Data from each compound are presented as mean \pm standard deviation. The Student's *t*-test was used for comparisons between treated and untreated groups. *P* values <0.01 and <0.001 were considered statistically highly significant and very highly significant, respectively. Two-way analysis of variance was used for testing the effect of the interaction of two compounds. *P* values <0.05 were considered statistically significant for a positive effect on the interaction of two compounds.

3. Results

3.1. Cell viability

Cell survival was evaluated after three days of treatment in various concentrations of the 16 compounds (Fig. 1). Under the tested concentrations, most of the compounds appear to be non-toxic or slightly toxic to the cells. On the contrary, for atazanavir, lopinavir/ritonavir, nelfinavir, saquinavir, efavirenz, and emodin, a dramatic cytotoxic effect was observed. The CC_{50} value of each compound was listed in Table 1.

3.2. Screening the 16 test compounds for antiviral activity

NTU156-infected fcwf-4 cells displayed a typical CPE characterized by generalized multinucleated giant cells (Fig. 2). Based on the viability results, the highest concentration of every test compound showing less than 5% cytotoxicity was chosen and further tested for antiviral effects. Among the 16 compounds tested, GNA and nelfinavir significantly inhibited the number of CPE foci induced by infection with FCoV when compared with the untreated groups (Table 1 and Fig. 3A). The IC_{50} values of GNA and nelfinavir at MOI 0.01 (2000 PFU/mL) were 0.0088 nM and 8.19 μM , respectively (Table 1). GNA was more potent than nelfinavir at inhibiting FCoV replication.

3.3. Concentration-dependence of antiviral effects

To further determine whether the antiviral effects are concentration-dependent, four concentrations of nelfinavir and GNA were tested. Only the highest concentration (9.41 μM) of nelfinavir significantly blocked the induction of CPE foci ($P < 0.001$) (Fig. 3B). GNA, in contrast, showed strong inhibitory activities at all four concentrations, and the antiviral effect was concentration-dependent (Fig. 3B).

3.4. Combined use of nelfinavir and GNA in fcwf-4 cells

The experiments described above establish an effect of GNA and nelfinavir on the preentry and entry steps of viral infection. Next, we sought to determine whether the compounds are effective at postentry steps in an environment of high viral load, as found in FIP cats (Kipar et al., 2006). To mimic the target cells present in the tissue of diseased animals, we preinfected cells before treating them with the antiviral compounds. The fcwf-4 cells were infected at a MOI 0.1 (20,000 PFU/mL), and the cells were then treated with either one or both antiviral compounds for 48 h. MTT and plaque assays were simultaneously performed to examine the cell damage caused by viral replication and the presence of infectious virus in the culture supernatant. Despite a lack of significant cell damage at 48 h postinoculation (Supplementary data 1), the results of

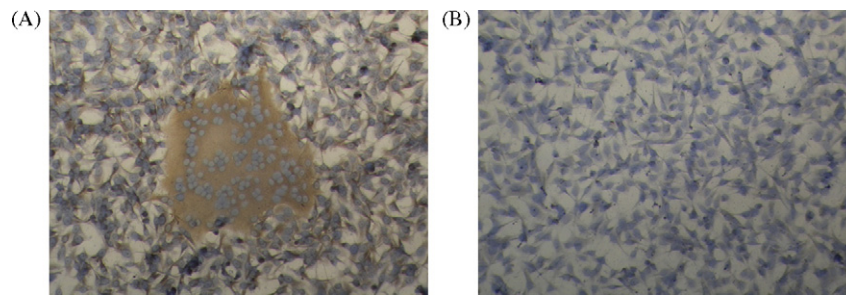


Fig. 2. Growth of FCoV in fcwf-4 cells. A typical CPE is characterized by generalized multinucleated giant cells (A) appearing by 15 h postinfection; mock-infected cells are shown in (B). The presence of FCoV antigen was confirmed by immunohistochemistry (200 \times).

the plaque assay revealed that treatment with either GNA or nelfinavir alone at their highest concentrations, 0.48 nM and 9.41 μ M, respectively (Fig. 4 and supplementary data 2) did not completely inhibit FCoV replication. However, a synergistic antiviral effect was observed when both agents were added simultaneously. FCoV replication was completely blocked under six combinations, i.e., nelfinavir (9.41 μ M) and GNA (0.12–0.48 nM), or nelfinavir (6.27–9.41 μ M) and GNA (0.48 nM) (Fig. 4 and supplementary data 2). To rule out possible cytotoxicity due to the combination of the two compounds, MTT assays were performed under all the effective combinations observed; no cytotoxicity was found for any of the effective combinations (data not shown).

4. Discussion

When tested on cells infected at a low concentration of FCoV, nelfinavir and GNA proved to be effective at inhibiting viral replication. Nelfinavir is an inhibitor of the HIV-1 protease with strong *in vivo* activity. This drug is safe and widely used in HIV-infected patients (Lewis et al., 1997). The antiviral effect of nelfinavir against coronaviruses has been investigated in SARS-CoV infection (Yamamoto et al., 2004). Similar to the finding that nelfinavir inhibits SARS-CoV strain FFM-1, this compound was found to significantly block replication of FCoV. The IC_{50} of nelfinavir on SARS-CoV

was 0.048 μ M. This concentration appears to be lower than on our FCoV (8.19 μ M). The difference in efficacy might result from low amino acid sequence homology (44%) of the two viruses (SARS-CoV FRA, GenBank accession number: AY310120; FCoV NTU156, accession number: GQ152141) in the putative 3C-like protease (3CLpro), i.e., the target molecule of nelfinavir. Furthermore, of the 18 residues in 3CLpro of SARS-CoV that have been identified to interact with several HIV protease inhibitors including nelfinavir (Rajnarayanan et al., 2004), only 7 were identical to the corresponding residues of 3CLpro of FCoV. This might also be the main factor that contributes to the high SI data of Nelfinavir on SARS-CoV in comparison to FCoV besides the difference of cells used in both assays. Our clinical trials using nelfinavir as a mean to control FCoV infection revealed that no obviously side effects, e.g., diarrhea or nausea, as occasionally seen in human HIV patients taking this agent, was observed in the 9 clinical healthy FCoV-shedding cats receiving the dosage 6.25 mg to 50 mg/kg/day per os. These preliminary findings raise a hope for using it in future clinical practice. This is the first study to demonstrate that an inhibitor of HIV-1 protease activity can effectively block the replication of an FCoV.

GNA, a carbohydrate-binding agent, exhibits its antiviral effect by binding to the two glycosylated envelope glycoproteins, the spike and membrane protein of coronaviruses (van der Meer et al., 2007b). Recent studies *in vitro* have shown that GNA strongly

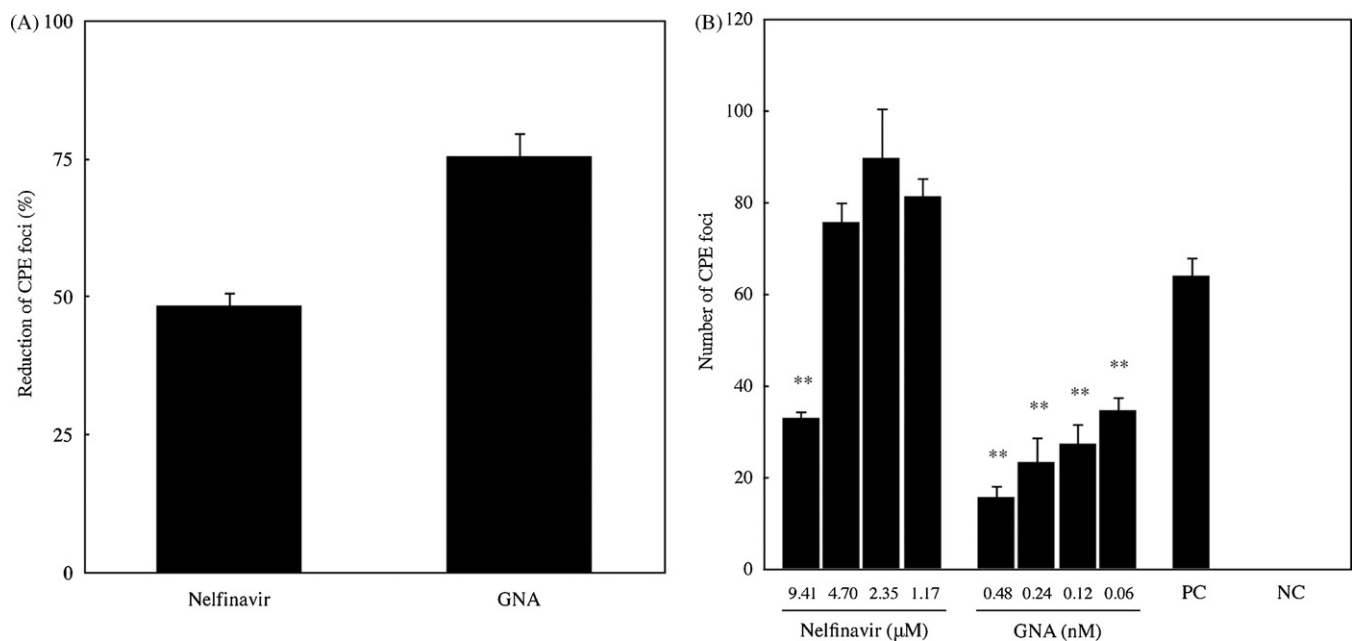


Fig. 3. Antiviral activity of nelfinavir and GNA. At 15 h postinfection, CPE foci were counted under an inverted microscope. (A) Reduction of CPE foci in FCoV-infected cells following treatment with nelfinavir or GNA. (B) Nelfinavir significantly blocked the induction of CPE foci only at the highest concentration tested, whereas GNA showed strong inhibitory activity in a concentration-dependent manner. Statistical analyses of antiviral activity were performed using Student's *t*-test. ** $P < 0.001$, compared with positive control. PC, positive control; NC, negative control. All samples were analyzed in triplicate.

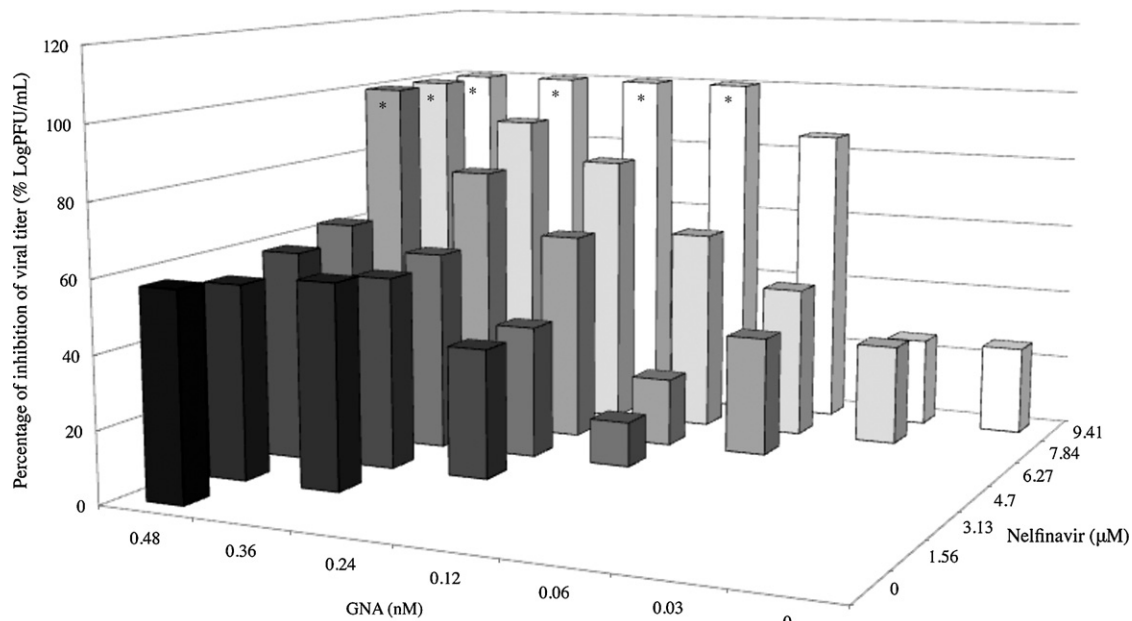


Fig. 4. Synergistic antiviral effects of nelfinavir and GNA in combination. To mimic the target cells present in the tissue of FCoV-infected cats, fcwf-4 cells were infected with MOI 0.1 of FCoV (20,000 PFU/mL) and then treated with a range of concentrations of each drug alone or in combination for 48 h. The mean plaque reduction rate in different combinations. The presence of the infectious virus in the culture supernatant was measured by plaque assay. When treating with the highest concentration of one of the agents and in combination with the other, a complete blockage was observed. All samples were analyzed in triplicate. Statistical analyses of synergism of antiviral activity were performed using two-way analysis of variance. *A synergistically antiviral effect ($P < 0.05$).

inhibits FCoV of serotypes I and II at the preentry step (van der Meer et al., 2007a). Our cell viability tests showed that GNA could be used safely with fcwf-4 cells over a broader concentration range than nelfinavir (Table 1). This agent showed antiviral activity against the local type II FCoV strain in a concentration-dependent manner when applied at the preentry step (Fig. 3B).

The aim of this study was to search for effective anti-FCoV agents for future clinical applications. Therefore, we also examined the antiviral effect of these compounds at postentry steps in cells preinoculated with high concentrations of virus (MOI 0.1) in order to mimic the environment of infected cells inside the body of a FIP cat. Under these conditions, the two effective compounds lost their anti-FCoV activity when used by themselves. This was surprising, given that the antiviral effect of nelfinavir has been demonstrated at the postentry step of SARS-CoV infection (Yamamoto et al., 2004). The antiviral activity of nelfinavir could not be assayed further due to the cytotoxicity of the compound at higher concentrations (Table 1). The fact that GNA lost its antiviral effect in cells preinoculated with FCoV was not unexpected because this compound works primarily at the step of viral attachment (Keyaerts et al., 2007). When used together, the two compounds were effective at inhibiting production of infectious FCoV. This synergistic effect may reflect the compounds' complementary mechanisms of action, with nelfinavir affecting postentry steps and GNA affecting the preentry step.

We speculate that GNA and nelfinavir will be used clinically as follows: (i) for prevention of FIP in high-risk groups, e.g., full-sibling littermates of FIP kittens (Addie et al., 2004) and the FCoV-shedding population living in multiple-cat households and (ii) for treatment of early-stage FIP, since FIP cats always show chronic secondary fever accompanied by an increase of viral RNA in plasma and/or white blood cells before death (de Groot-Mijnes et al., 2005). For cases of FIP diagnosed early, the administration of effective antiviral agents may provide a chance to prevent secondary viremia, which triggers the fatal stage of this disease. As systemic application of GNA might provoke antibody responses leading to inactivity of the compound, and the primary antibody response generally appears

about 2 weeks after the antigen stimulation (Murphy et al., 2008), the future therapeutic protocol will need to be designed to administer GNA in an efficient dose within the first 2 weeks, together with other effective antiviral agents like nelfinavir found in this study and some others currently under testing. With proper combination of effective agents targeting at different stages of viral replication, a cocktail therapy like treating HIV human patients may be available in the near future for cats suffering from FIP. The known side effect for nelfinavir is mild diarrhea, as previously observed in HIV patients (Kaldor et al., 1997), whereas no side effects for GNA have been reported.

Since FIP is an immunopathogenic disease, treatments aimed at the control of viral replication alone will not be sufficient. Successful supplementary treatments may include antiviral drugs, interferon, and immunosuppressive therapy, and perhaps cytokine antagonists, such as the tumor necrosis factor alpha (TNF-alpha) antagonist. This antagonist has been shown to reduce mortality in another immune-mediated disease, dengue virus infection (Atrasheuskaya et al., 2003). This reagent may also be effective in FIP cats; given that an increased level of TNF-alpha has been noted in FIP cats (Takano et al., 2007a,b).

In this study, we demonstrate the synergistic antiviral effect of GNA and nelfinavir on the inhibition of FCoV replication in infected cells. Our results suggest that the combined use of these two agents is a potential therapy for preventing and treating FIP cats, as long as diagnosis can be made early.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.06.010.

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