Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Research in Veterinary Science 144 (2022) 27–33

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

In vitro antiviral effects of GS-441524 and itraconazole combination against feline infectious peritonitis virus

Tomoyoshi Doki, Ken Takahashi, Nobuhisa Hasegawa, Tomomi Takano *

Laboratory of Veterinary Infectious Disease, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

ARTICLE INFO

Keywords:
Feline infectious peritonitis virus
Coronavirus
Drug combination

ABSTRACT

Feline infectious peritonitis virus (FIPV: virulent feline coronavirus) causes a fatal disease called feline infectious peritonitis (FIP) in wild and domestic cat species. Recent studies identified several antiviral drugs that are effective against FIPV. Drug combination is one of the important strategies in the development of novel treatments for viral infections. GS-441524, a nucleoside analog, and itraconazole, a triazole antifungal drug, have been reported that have antiviral effect against FIPV. This study aims to investigate whether the combination of GS-441524 and itraconazole has synergic antiviral effect against FIPV. The antiviral effect was measured by plaque reduction assay using felis catus whole fatus-4 cell. The plaque reduction of GS-441524 against type I FIPVs increased as the concentration of itraconazole increased. The similar result was obtained for type II FIPV. In addition, the calculated combination index (CI) demonstrated that there was a strong synergy between GS-441524 and itraconazole. It is concluded that the combination of GS-441524 and itraconazole may enhance the individual effect of each drug against replication of type I FIPVs and may contribute to development more effective treatment strategy for FIP.

1. Introduction

Feline coronavirus (FCoV) belongs to the Order *Nidovirales,* family *Coronaviridae,* subfamily *Orthocoronavirinae,* genus *Alphacoronavirus,* species *Alphacoronavirus 1*(ICTV, 2019). FCoVs are classified into two biotypes, feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV). Although FECV is avirulent, FIPV causes a fatal disease called feline infectious peritonitis (FIP) in wild and domestic cat species. FIP typically manifests effusion accumulation and granuloma formation on the surface of several organs (Pedersen, 2014). The mortality rate of cats with FIP is high.

The FCoV virion is mainly composed of nucleocapsid (N), envelope (E), membrane (M), and peplomer spike (S) proteins. FCoVs are classified into two serotypes, type I and II FCoV, based on differences in the sequence of S protein and the 5′-region of the genome (Jaimes and Whittaker, 2018; Tekes and Thiel, 2016). Several serological and genetic surveys have shown that type I FCoV is dominant and that most cases of FIP are caused by type I FIPV infection (Addie et al., 2003; Hohdatsu et al., 1992; Kummrow et al., 2005).

Recent studies identified antiviral drugs that are effective against FIPV. GS-441524 is a nucleoside analog core of GS-5734 (remdesivir). It becomes phosphorylated by nucleoside triphosphate inside a cell to suppress the activity of viral RNA-dependent RNA polymerase. Because GS-5734 is hydrolyzed in the gastrointestinal tract, oral administration of GS-5734 is not approved (Sun, 2020). On the other hands, it has been reported that GS-441524 showed effective oral bioavailability (Xie and Wang, 2021; Yan et al., 2021). Therefore, GS-441524 is expected as oral antiviral drug against coronavirus. A study demonstrated *in vitro* that GS-441524 is not only effective in suppressing replication of FIPV but can also be used to treat cats suffering from naturally acquired FIP (Dickinson et al., 2020; Murphy et al., 2018; Pedersen et al., 2019). However, some do not respond to treatment with GS-441524, and some relapse of FIP after the treatment. Animals that responded poorly to GS-441524 may require higher doses to achieve treatment effect (Pedersen et al., 2019). Also, to treat cat with dry or neurological form of FIP may require higher doses than wet form because the blood-ocular and blood-brain barriers inhibit the diffusion of GS-441524 (Pedersen et al., 2019; Dickinson et al., 2020).

Itraconazole (ICZ) is a triazole antifungal drug and is used against feline and canine fungal infections. Studies suggest that ICZ also has an antiviral effect. We previously demonstrated that ICZ strongly inhibited type I FIPV replication (Doki et al., 2020; Takano et al., 2019a). ICZ

* Corresponding author.
E-mail addresses: doki@vmas.kitasato-u.ac.jp (T. Doki), dv20004@st.kitasato-u.ac.jp (N. Hasegawa), takanot@vmas.kitasato-u.ac.jp (T. Takano).

https://doi.org/10.1016/j.rvsc.2022.01.005
Received 21 May 2021; Received in revised form 28 December 2021; Accepted 5 January 2022
Available online 7 January 2022

inhibits the endosomal pathway of cells; specifically, it inhibits pathways that are downstream of late endosome, which plays an important role in the entry of type I FIPV into cells (Takano et al., 2019b).

A combination of two antiviral drugs with independent mechanisms of action may elicit a superior antiviral effect than what can be achieved individually. In fact, combination antiviral therapy is used as a highly effective strategy against human immunodeficiency virus (HIV) and hepatitis C virus (HCV) (Atta et al., 2019; Cohen et al., 2016; Kohli et al., 2014; Palella et al., 1998; Rodger et al., 2016). The combination of GS-441524 and ICZ may also have synergistic or additive antiviral interaction against FIPV since they elicit their effects by different mechanisms of action. In this study, we examined whether the combination of GS-441524 and ICZ (GS-ICZ combination) shows a greater antiviral effect than a single drug alone.

2. Material and method

2.1. Cell cultures and viruses

fcwf-4 cells (kindly supplied by Dr. M. C. Horzinek of the State University of Utrecht) were grown in Eagle's minimum essential medium containing 50% L-15 medium, 5% fetal calf serum (FCS),100 U of penicillin per mL, and 100 μg of streptomycin per mL. The fcwf-4 cells were cultured for at least 70 passages. Type I FIPV KU-2 was isolated in our laboratory. Type I FIPV Black was kindly provided by Dr. J. K. Yamamoto from the University of Florida. Type II FIPV 79–1146 was supplied by Dr. M. C. Horzinek of the State University Utrecht, the Netherlands. These viruses were grown in fcwf-4 cells at 37 °C.

2.2. Compounds

GS-441524 was purchased from Carbosynth Limited(Berkshire, United Kingdom). GS-441524 was prepared as 2 mM stocks in dimethyl sulfoxide (DMSO). ICZ was purchased from Janssen Pharma-central K. K. (Tokyo, Japan). ICZ was dissolved as 10 mM stocks in 40% (*w/v*) hydroxypropyl-beta-cyclodextrin (HβCD) solution.

2.3. Cytotoxic effects of compounds

The fcwf-4 cells were seeded on 96-well plates. The cells were treated with ICZ for 24 h at the indicated concentrations. The cells were washed three times and the serial diluted GS-441524 were added to the wells. After incubation for 24 h, the culture supernatants were removed. WST-8 solution (Kishida Chemical, Osaka, Japan) was added, and the cells were returned to the incubator for 1 h. The absorbance of formazan produced was measured at 450 nm using a 96-well spectrophotometric plate reader (Thermofisher scientific inc., Waltham, MA, U.S.A.) as described by the manufacturer. Percentage cytotoxicity was calculated using the following formula: Cytotoxicity (%) = [(OD of compound-untreated cells−compound-treated cells)/(OD of compound-untreated cells)] × 100.

2.4. Plaque reduction assay

Cells were treated with GS-441524 and ICZ according to protocols previously described by Murphy et al. and Takano et al., respectively (Murphy et al., 2018; Takano et al., 2019a). In briefly, confluent fcwf-4 cell monolayers were treated with ICZ at the indicated concentrations in 24-well multi-plates at 37 °C for 24 h. the cells were washed and the virus (1×10^2 PFU/0.1 mL/well) was adsorbed into the cells at 37 °C for 1 h. After washing, the cells were cultured in carboxymethylcellulose (CMC)-MEM containing GS-441524. The cell monolayers were incubated at 37 °C for 48 h, fixed and stained with 1% crystal violet solution containing 10% buffered formalin, and the resulting plaques were then counted. The plaque reduction percentage was calculated using the following formula: Plaque reduction percentage (%) = [(plaque number of compound-treated cells)/(plaque number of compound-untreated cells)] × 100.

2.5. Data analysis

Data collected from the plaque reduction assay were analyzed using Compusyn software (ComboSyn, Inc., https://www.combosyn.com/index.html). Data were excluded according to the protocol defined by compusyn software if the concentration of GS-441524 and ICZ resulted in the following conditions: 1) plaque reduction of 100%, and 2) plaque reduction of less than 25% (*i.e.*, ineffective against FIPV) (Chou, 2014; ComboSyn Inc., 2017). The combination index (CI) was calculated and categorized according to the classification proposed by Cho et al. (Chou, 2006; Chou and Talalay, 1984).

3. Result

3.1. Cytotoxicity of GS-ICZ combination against fcwf-4 cells

The cytotoxicity of GS-441524 alone, ICZ alone, and GS-ICZ combination against fcwf-4 cells was quantified. None of the treatment groups showed a dose-dependent increase in cytotoxicity (Fig. 1). The highest level of cytotoxicity (16.7%) was observed at the combination of 6.3 μM GS-441524 and 2.5 μM ICZ.

3.2. Effect of GS-ICZ combination on replication of type I and II FIPV

The fcwf-4 cells were treated with ICZ for 24 h and were subsequently exposed to FIPV for 1 h before GS-441524 was added to the cell culture. The resulting plaques were counted after a certain period of incubation to calculate the plaque reduction percentage. As shown in Fig. 2, plaque reduction of type I FIPV KU-2 by GS-441524 and ICZ alone increased in a dose-dependent manner. Specifically, GS-441524 alone resulted in 100% plaque reduction when administered at 6.3 and 3.1 μM. IC50 and IC90 of GS-441524 alone against type I FIPV KU-2 were 1.4 and 2.8 μM, respectively. For ICZ alone, the maximum plaque reduction was 87.1% at 20 μM. IC50 and IC90 of ICZ alone were 1.0 and 39.0 μM, respectively. For GS-441524-ICZ combination, plaque reduction of GS-441524 increased as the dose of ICZ increased. This dose-dependent increase in plaque reduction was not observed in the vehicle control.

For type I FIPV Black, similar results were obtained with the drug combination and the single drug alone (Fig. 3A). GS-441524 alone resulted in 100% plaque reduction of type I FIPV Black when administered at 6.3 and 3.1 μM, with IC50 and IC90 of 1.3 and 2.8 μM, respectively. ICZ alone resulted in 100% plaque reduction at 20 μM, with IC50 and IC90 of 0.3 and 18.1 μM, respectively. Plaque reduction of type II FIPV 79–1146 increased as the dose of GS-441524 increased, with 100% plaque reduction at 6.3 μM. IC50 and IC90 of GS-441524 alone were 0.8 and 3.3 μM, respectively (Fig. 3B). ICZ did not result in a dose-dependent increase of plaque reduction against type II FIPV 79–1146. For ICZ alone, the maximum plaque reduction of type II FIPV 79–1146 was 34.6% at 20 μM. IC50 and IC90 could not be calculated. For GS-441524-ICZ combination, plaque reduction by GS-441524 increased when the concentration of ICZ was at 20 μM.

3.3. Combination index (CI) and drug reduction index (DRI)

The CI was calculated to determine the interaction of GS-441524 and ICZ. CI is used to determine a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1). For both type I FIPV KU-2 and Black, the CI tended to decrease as the fractional inhibition (FI) increased (Tables 1 and 2, Fig. 4). Specifically, the combination of GS-441524 and ICZ was highly effective with FI > 0.9 for both type I FIPV KU-2 and Black, indicating very strong or strong synergism.

DRI was also calculated for GS-441524 and ICZ. DRI is a measure of

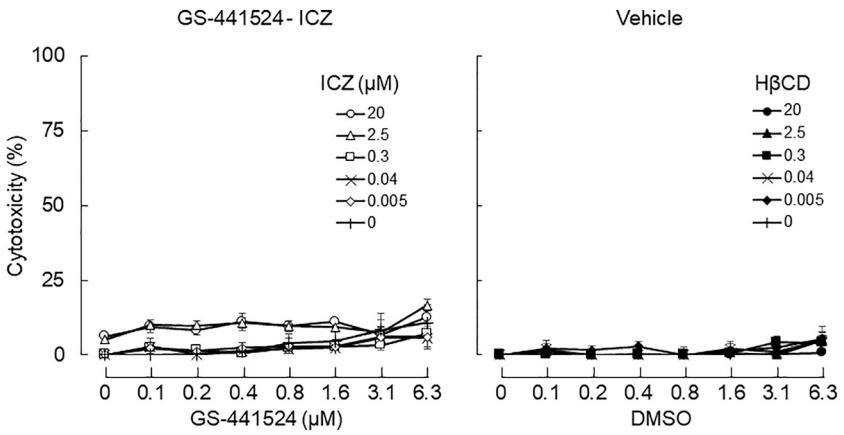

Fig. 1. Cytotoxic effects of GS-ICZ combination on *Felis catus* whole fetus-4 cells (fcwf-4 cells). The fcwf-4 cells were seeded on 96-well plates. The cells were treated with ICZ for 24 h at the indicated concentrations. The cells were washed and the serial diluted GS-441524 were added to the wells. After incubation for 24 h, the culture supernatants were removed. WST-8 solution was added, and the cells were incubated for 1 h. The absorbance of formazan produced was measured at 450 nm. Percentage cytotoxicity was calculated using the following formula: Cytotoxicity (%) = [(OD of compound-untreated cells−compound-treated cells)/(OD of compound-untreated cells)] × 100. The results are shown as mean ± SE. Data represent three independent experience.

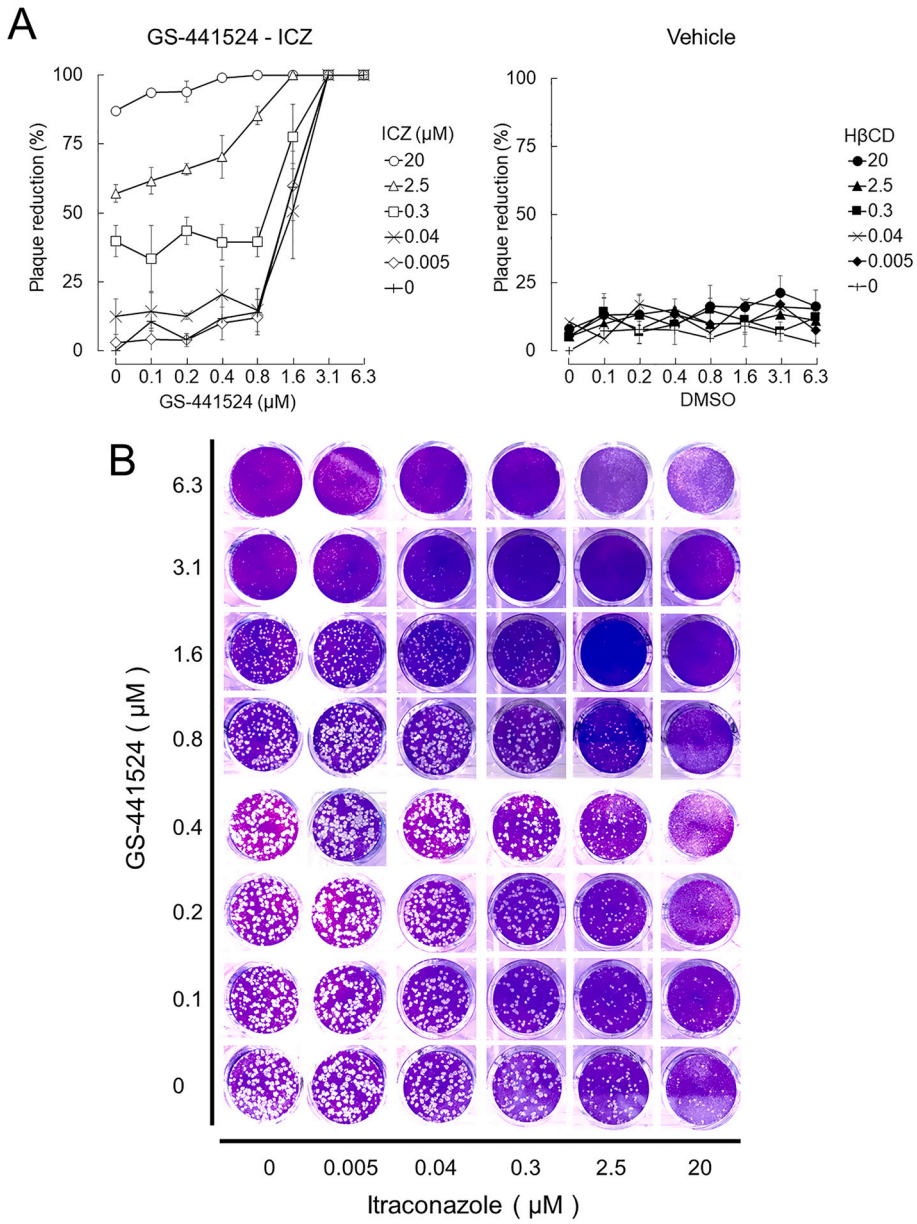

Fig. 2. The effects of GS-ICZ combination on type I FIPV KU-2. The fcwf-4 cells were pretreated with ICZ for 24 h. After viral adsorption, the cells were cultured in CMC-MEM containing GS-441524. The cells were fixed and stained with 1% crystal violet and the resulting plaques were then counted. The plaque reduction percentage was calculated using the following formula: Plaque reduction percentage (%) = [(plaque number of compound-treated cells)/(plaque number of compound-untreated cells)] × 100. (A) Plaque reduction in treated with GS-441524 and ICZ. The results are shown as mean ± SE. Data represent three independent experience. (B) Crystal violet stain of FIPV infected fcwf-4 cells treated with GS-441524 and ICZ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

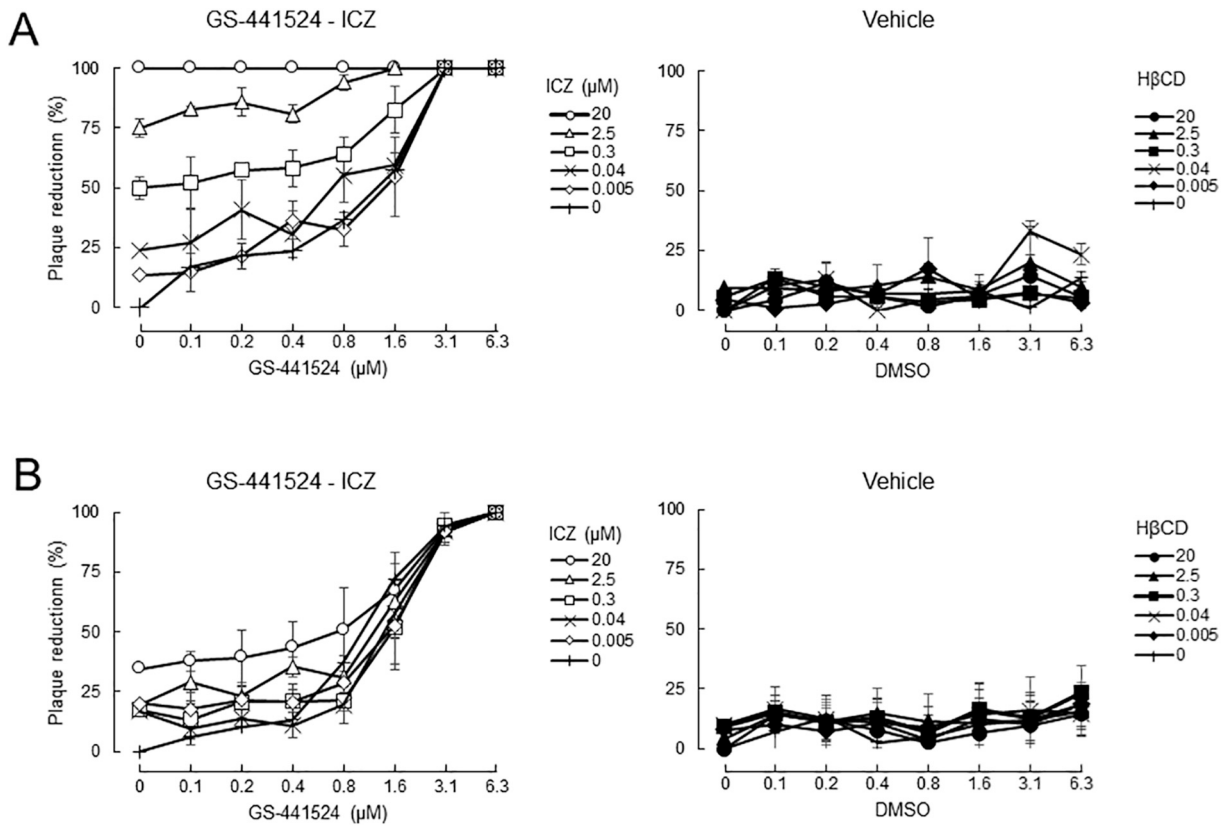

Fig. 3. The effects of GS-ICZ combination on type I and II FIPV. The fcwf-4 cells were pretreated with ICZ for 24 h. After viral adsorption, the cells were cultured in CMC-MEM containing GS-441524. The cells were fixed and stained with 1% crystal violet and the resulting plaques were then counted. The plaque reduction percentage was calculated using the following formula: Plaque reduction percentage (%) = [(plaque number of compound-treated cells)/(plaque number of compound-untreated cells)] × 100. The results are shown as mean ± SE. Data represent three independent experience. (A) type I FIPV Black. (B) type II FIPV 79–1146. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
The combination index and drug reduction index of GS-441524-ICZ combination for type I FIPV KU-2.

Dose		Fractional inhibition	Combination index	Estimated dose[a]		Drug reduction index		Description
ICZ	GS-441524			ICZ	GS-441524	ICZ	GS-441524	
20	0.4	0.991	0.071	2689.5	6.3	134.5	15.7	Very strong synergism
20	0.2	0.940	0.260	99.5	3.4	5.0	17.0	Strong synergism
20	0.1	0.937	0.249	91.2	3.3	4.6	33.4	Strong synergism
2.5	0.8	0.853	0.458	18.6	2.5	7.4	3.1	Synergism
2.5	0.4	0.703	0.827	4.1	1.9	1.6	4.7	Synergism
2.5	0.2	0.659	0.978	2.9	1.7	1.2	8.7	Nearly additive
2.5	0.1	0.616	1.244	2.1	1.6	0.8	16.5	Moderate antagonism
0.3	1.6	0.777	0.797	7.8	2.1	26.1	1.3	Moderate synergism
0.3	0.8	0.395	1.294	0.5	1.2	1.5	1.6	Moderate antagonism
0.3	0.4	0.393	0.981	0.5	1.2	1.5	3.1	Nearly additive
0.3	0.2	0.435	0.644	0.6	1.3	2.0	6.5	Synergism
0.3	0.1	0.333	1.109	0.3	1.1	1.0	11.4	Moderate antagonism
0.04	1.6	0.506	1.158	1.0	1.4	24.7	0.9	Moderate antagonism
0.005	1.6	0.598	0.996	1.9	1.6	371.9	1.0	Nearly additive

[a] Dose required to produce fractional inhibition.

the dose needed for the single drug alone to achieve the same FI of the drug combination. DRI > 1 indicates favorable dose reduction, and DRI < 1 indicates unfavorable dose reduction. For type I FIPV KU-2, DRI of ICZ and GS-441524 ranged between 0.8 and 371.9 and 0.9–33.4, respectively (Tables 1 and 2, Fig. 4). Similarly, DRI of ICZ and GS-441524 for type I FIPV Black ranged between 1.3 and 88.0 and 0.8–54.0, respectively. DRI of ICZ and GS-441524 needed to achieve FI > 0.9 was 4.6–134.5 and 15.7–33.4, respectively.

4. Discussion

Drug combination is one of the important strategies in the development of novel treatments for viral infections. Antiretroviral therapy (ART) is an example where multiple anti-HIV drugs are used in combination to effectively treat HIV infection. ART resulted in a reduction of the incidence of acquired immuno-deficiency syndrome (AIDS) and prolonged the overall survival of HIV-infected patients (Poorolajal et al., 2016). Similarly, the combination of interferon and multiple anti-HCV drugs were shown to be superior for the treatment of HCV infection

Table 2
The combination index and drug reduction index of GS-441524-ICZ combination for type I FIPV Black.

Dose		Fractional inhibition	Combination index	Estimated dose[a]		Drug reduction index		Description
ICZ	GS-441524			ICZ	GS-441524	ICZ	GS-441524	
2.5	0.8	0.939	0.100	48.5	16.5	19.4	20.7	Strong synergism
2.5	0.4	0.806	0.652	4.4	4.8	1.8	12.0	Synergism
2.5	0.2	0.857	0.320	8.6	6.8	3.4	33.9	Synergism
2.5	0.1	0.827	0.458	5.7	5.5	2.3	54.8	Synergism
0.3	1.6	0.823	0.355	5.4	5.3	18.0	3.3	Synergism
0.3	0.8	0.638	0.703	0.9	2.1	3.0	2.7	Moderate synergism
0.3	0.4	0.583	0.735	0.6	1.7	2.0	4.3	Moderate synergism
0.3	0.2	0.573	0.663	0.6	1.7	1.8	8.3	Synergism
0.3	0.1	0.519	0.882	0.4	1.3	1.2	13.5	Slight synergism
0.04	1.6	0.594	0.953	0.6	1.8	16.2	1.1	Nearly additive
0.04	0.8	0.552	0.609	0.5	1.5	11.8	1.9	Synergism
0.04	0.4	0.308	1.232	0.1	0.6	1.8	1.5	Moderate antagonism
0.04	0.2	0.408	0.472	0.2	0.9	4.1	4.4	Synergism
0.04	0.1	0.271	0.963	0.1	0.5	1.3	4.9	Nearly additive
0.005	1.6	0.542	1.101	0.4	1.5	88.0	0.9	Moderate antagonism
0.005	0.8	0.325	1.332	0.1	0.6	16.9	0.8	Moderate antagonism
0.005	0.4	0.362	0.589	0.1	0.7	22.9	1.8	Synergism

[a] Dose required to produce fractional inhibition.

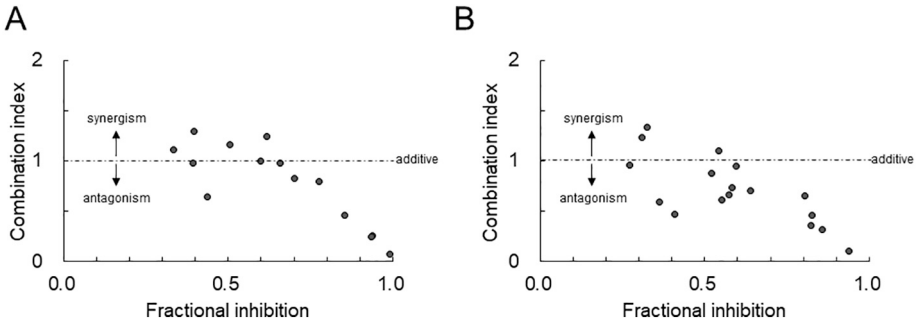

Fig. 4. FI-CI plots of GS-ICZ combination. The X-axis and Y-axis indicate fractional inhibition and combination index, respectively. (A) type I FIPV KU-2. (B) type I FIPV Black.

(Kohli et al., 2014). For coronavirus, combinations of anti-viral drugs against severe acute respiratory syndrome coronavirus (SARS-CoV), SARS-CoV 2, and Middle East respiratory syndrome coronavirus are being examined (Arabi et al., 2020; Caly et al., 2020; Han et al., 2021; Tai, 2007). Similarly, the synergistic antiviral effect of *Galanthus nivalis* agglutinin and nelfinavir has been examined against FIPV (Hsieh et al., 2010). However, there are no studies on the combination of GS-441524 and ICZ. In the present study, we examined whether the combination of GS-441524 and ICZ enhances the effects of individual drugs alone against FIPV *in vitro*.

Anti-viral mechanisms of action against FIPV have been well-characterized for both GS-441524 and ICZ (Murphy et al., 2018; Takano et al., 2019a, 2019b). GS-441524 inhibits RNA-dependent RNA polymerase, while ICZ prevents from releasing FIPV genome from endosome to cytosol. Thus, they elicit an antiviral effect through distinct mechanisms of action. As such, we hypothesized that GS-441524 and ICZ can be combined as an antiviral treatment against FIPV. Cells were pretreated with ICZ and were subsequently exposed to FIPV. After FIPV was adsorbed by the cells, GS-441524 was added to the cell culture to determine the antiviral effect of the combination therapy. We demonstrated that GS-ICZ combination was effective against type I FIPVs; specifically, we demonstrated that plaque reduction of GS-441524 increased as the concentration of ICZ increased. GS-441524 (1.6 μM) and ICZ (2.5 μM) alone did not result in 100% plaque reduction of type I FIPVs. However, 100% plaque reduction was achieved when the same concentration of drugs was combined. In other words, the combination was more effective against FIPV than the single drug alone. In addition, we calculated the CI and demonstrated that there was a strong synergy between GS-441524 and ICZ at concentrations that achieved FI $>$ 0.9. These findings suggest that the combination of GS-441524 and ICZ may enhance the individual effect of each drug against replication of type I FIPVs.

Twice dosage of 10 mg/kg of ICZ orally produced peak blood levels of 4.8 $\pm$ 1.8 μM in cats (Boothe et al., 1997). The blood level of GS-441524 in cats administrated 5 mg/kg of GS-441524 subcutaneously was sustained above 1 μM for 12 h (Murphy et al., 2018). The results of the plaque reduction assay and CI suggest that more than 0.4 μM GS-441524 and more than 2.5 μM ICZ are required to achieve synergistic effects against type I FIPVs. These *in vitro* concentrations are lower than the blood levels reported by pharmacokinetic studies and possible to be achieved *in vivo*. On the other hand, ICZ is known to be difficult to penetrate the blood-ocular and blood-brain barriers (Felton et al., 2014). In rats, the concentration of ICZ in the brain is ten times lower than that in plasma (Miyama et al., 1998). It is suggested that the effects of the treatment of FIP using ICZ-GC combination remain to be evaluated *in vivo*.

The effect of ICZ against replication of type II FIPV is inferior compared with the effect against type I FIPVs (Takano et al., 2019a; Takano et al., 2017). This is attributed to the fact that the mechanism of cellular entry through the endosomal pathway differs for type I and type II FIPVs (Takano et al., 2019b). In accordance with previous studies, we also demonstrated that plaque reduction in type II FIPV 79–1146 did not increase in an ICZ dose-dependent manner. In fact, we demonstrated that the maximum effect achieved was limited to 50%. On the other hand, the combination of GS-441524 and ICZ was effective in increasing plaque reduction by GS-441524. This indicates that even though ICZ is

less effective for type II FIPVs, it becomes more effective when combined with GS-441524. Between the two serotypes of FCoVs, type I FCoV is more prevalent (Addie et al., 2003; Hohdatsu et al., 1992; Kummrow et al., 2005). However, the incidence of type II FCoV infection is higher in Japan and Taiwan compared with European countries (Hohdatsu et al., 1992; Lin et al., 2009). Thus, in countries where type II FCoV infection has been reported, the combination of GS-441524 and ICZ may be an effective treatment strategy against FIP.

IC50 of GS-441524 and ICZ against type I FIPVs were equivalent. In contrast, while IC90 of GS-441524 alone against type I FIPVs was 2.0–2.2 times higher compared with IC50, IC90 of ICZ was 39.0–60.3 times higher compared with IC50. This finding indicates that a high dose of ICZ may be required for the treatment of FIP. However, when combined with GS-441524, the same antiviral effect could be achieved using 0.7–21.7% of ICZ concentration as measured by DRI. Similarly, only 3.0–6.4% of GS-441524 concentration was needed to achieve the same effect. These findings indicate that the dose of GS-441524 and ICZ may be reduced when the two drugs are combined to have the same treatment effect against FIP. Although GS-441524 is currently unavailable for use in animals, it will likely be very costly when it becomes commercially available. Thus, long-term treatment with GS-441524 will likely be challenging from the perspective of cost. Thus, the combination therapy may be more advantageous as the dose of GS-441524 can be reduced to continue the treatment. The doses of GS-441524 and ICZ indicate 100% plaque reduction were excluded from the calculation of DRI. The DRIs with high FI values were extrapolated and may differ from the original rate of drug reduction. Further studies are needed to evaluate DRI for FIP treatment.

We examined the cytotoxicity of GS-441524 and ICZ using fcwf-4 cells and demonstrated that the combination did not result in a significant increase in cytotoxicity. Thus, at the concentrations we tested, the combination did not enhance the cytotoxic effect on cells. Notably, there is possibility that high-dose ICZ cause damage to the liver. Although there is no evidence that ICZ causes direct damage to the liver in FIP cats, the side effects of the combination of ICZ with other drugs have not been fully elucidated (Doki et al., 2020; Kameshima et al., 2020). Furthermore, studies also demonstrated that when combined, ICZ affects the plasma concentration and clearance of individual drugs. The side effects of GS-441524 in cats have not been fully elucidated. While a study demonstrated that 5–10 mg/kg GS-441524 did not induce acute toxicity in cats, another study demonstrated that a repeated administration of GS-441524 (2–4 mg/kg, SID) over several weeks resulted in focal injection site reactions (Murphy et al., 2018; Pedersen et al., 2019). Thus, it is important to determine whether the administration of the combination of GS-441524 and ICZ increases the plasma concentration of each drug and causes more severe side effects in cats. Future studies should also be performed *in vivo* in FIP cats to determine the treatment effect of the combination of GS-441524 and ICZ in comparison to each drug alone.

In conclusion, we demonstrated that GS-441524 and ICZ act synergistically against FIPV *in vitro*. Future studies are needed to determine *in vivo* metabolism and pharmacokinetics, as well as the treatment effect of the combination therapy against FIP.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

Addie, D.D., Schaap, I.A.T., Nicolson, L., Jarrett, O., 2003. Persistence and transmission of natural type I feline coronavirus infection. J. Gen. Virol. 84, 2735–2744. https://doi.org/10.1099/vir.0.19129-0.

Arabi, Y.M., Asiri, A.Y., Assiri, A.M., Balkhy, H.H., Al Bshabshe, A., Al Jeraisy, M., Mandourah, Y., Azzam, M.H.A., Bin Eshaq, A.M., Al Johani, S., Al Harbi, S., Jokhdar, H.A.A., Deeb, A.M., Memish, Z.A., Jose, J., Ghazal, S., Al Faraj, S., Al Mekhlafi, G.A., Sherbeeni, N.M., Elzein, F.E., Al-Hameed, F., Al Saedi, A., Alharbi, N. K., Fowler, R.A., Hayden, F.G., Al-Dawood, A., Abdelzaher, M., Bajhmom, W., AlMutairi, B.M., Hussein, M.A., Alothman, A., 2020. Interferon Beta-1b and lopinavir–ritonavir for middle east respiratory syndrome. N. Engl. J. Med. 383, 1645–1656. https://doi.org/10.1056/nejmoa2015294.

Atta, M.G., De Seigneux, S., Lucas, G.M., 2019. Clinical pharmacology in HIV therapy. Clin. J. Am. Soc. Nephrol. 14, 435–444. https://doi.org/10.2215/CJN.02240218.

Boothe, D.M., Herring, I., Calvin, J., Way, N., Dvorak, J., 1997. Itraconazole disposition after single oral and intravenous and multiple oral dosing in healthy cats. Am. J. Vet. Res. 58, 872–877.

Caly, L., Druce, J.D., Catton, M.G., Jans, D.A., Wagstaff, K.M., 2020. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. Antivir. Res. 178, 104787 https://doi.org/10.1016/j.antiviral.2020.104787.

Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681. https://doi.org/10.1124/pr.58.3.10.

Chou, T.C., 2014. Frequently asked questions in drug combinations and the mass-action law-based answers. Synergy. https://doi.org/10.1016/j.synres.2014.07.003.

Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzym. Regul. 22, 27–55. https://doi.org/10.1016/0065-2571(84)90007-4.

Cohen, M.S., Chen, Y.Q., McCauley, M., Gamble, T., Hosseinipour, M.C., Kumarasamy, N., Hakim, J.G., Kumwenda, J., Grinsztejn, B., Pilotto, J.H.S., Godbole, S.V., Chariyalertsak, S., Santos, B.R., Mayer, K.H., Hoffman, I.F., Eshleman, S.H., Piwowar-Manning, E., Cottle, L., Zhang, X.C., Makhema, J., Mills, L. A., Panchia, R., Faesen, S., Eron, J., Gallant, J., Havlir, D., Swindells, S., Elharrar, V., Burns, D., Taha, T.E., Nielsen-Saines, K., Celentano, D.D., Essex, M., Hudelson, S.E., Redd, A.D., Fleming, T.R., 2016. Antiretroviral therapy for the prevention of HIV-1 transmission. N. Engl. J. Med. 375, 830–839. https://doi.org/10.1056/nejmoa1600693.

ComboSyn Inc., 2017. Data Entry Illustrations [WWW Document]. https://www.combosyn.com/dataEI.html. URL. https://www.combosyn.com/dataEI.html. accessed 5.14.21.

Dickinson, P.J., Bannasch, M., Thomasy, S.M., Murthy, V.D., Vernau, K.M., Liepnieks, M., Montgomery, E., Knickelbein, K.E., Murphy, B., Pedersen, N.C., 2020. Antiviral treatment using the adenosine nucleoside analogue GS-441524 in cats with clinically diagnosed neurological feline infectious peritonitis. J. Vet. Intern. Med. 34, 1587–1593. https://doi.org/10.1111/jvim.15780.

Doki, T., Toda, M., Hasegawa, N., Hohdatsu, T., Takano, T., 2020. Therapeutic effect of an anti-human-TNF-alpha antibody and itraconazole on feline infectious peritonitis. Arch. Virol. 1–10 https://doi.org/10.1007/s00705-020-04605-7.

Felton, T., Troke, P.F., Hope, W.W., 2014. Tissue penetration of antifungal agents. Clin. Microbiol. Rev. 27, 68–88.

Han, Y.J., Lee, K.H., Yoon, S., Nam, S.W., Ryu, S., Seong, D., Kim, J.S., Lee, J.Y., Yang, J. W., Lee, J., Koyanagi, A., Hong, S.H., Dragioti, E., Radua, J., Smith, L., Oh, H., Ghayda, R.A., Kronbichler, A., Effenberger, M., Kresse, D., Denicolò, S., Kang, W., Jacob, L., Shin, H., Shin, J. Il, 2021. Treatment of severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and coronavirus disease 2019 (COVID-19): a systematic review of in vitro, in vivo, and clinical trials. Theranostics. https://doi.org/10.7150/thno.48342.

Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., Koyama, H., 1992. The prevalence of types I and II feline coronavirus infections in cats. J. Vet. Med. Sci. 54, 557–562. https://doi.org/10.1292/jvms.54.557.

Hsieh, L.E., Lin, C.N., Su, B.L., Jan, T.R., Chen, C.M., Wang, C.H., Lin, D.S., Lin, C.T., Chueh, L.L., 2010. Synergistic antiviral effect of Galanthus nivalis agglutinin and nelfinavir against feline coronavirus. Antivir. Res. 88, 25–30. https://doi.org/10.1016/j.antiviral.2010.06.010.

ICTV, 2019. Virus Taxonomy: 2019 Release [WWW Document]. Int. Comm. Taxon. Viruses. URL. https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/219/nidovirales (accessed 5.6.21).

Jaimes, J.A., Whittaker, G.R., 2018. Feline coronavirus: insights into viral pathogenesis based on the spike protein structure and function. Virology 517, 108–121. https://doi.org/10.1016/j.virol.2017.12.027.

Kameshima, S., Kimura, Y., Doki, T., Takano, T., Park, C.-H., Itoh, N., 2020. Clinical efficacy of combination therapy of itraconazole and prednisolone for treating effusive feline infectious peritonitis. J. Vet. Med. Sci. 82 https://doi.org/10.1292/jvms.20-0049.

Kohli, A., Shaffer, A., Sherman, A., Kottilil, S., 2014. Treatment of hepatitis C a systematic review. JAMA. https://doi.org/10.1001/jama.2014.7085.

Kummrow, M., Meli, M.L., Haessig, M., Goenczi, E., Poland, A., Pedersen, N.C., Hofmann-Lehmann, R., Lutz, H., 2005. Feline coronavirus serotypes 1 and 2: seroprevalence and association with disease in Switzerland. Clin. Diagn. Lab. Immunol. 12, 1209–1215. https://doi.org/10.1128/CDLI.12.10.1209-1215.2005.

Lin, C.N., Su, B.L., Wang, C.H., Hsieh, M.W., Chueh, T.J., Chueh, L.L., 2009. Genetic diversity and correlation with feline infectious peritonitis of feline coronavirus type I and II: a 5-year study in Taiwan. Vet. Microbiol. 136, 233–239. https://doi.org/10.1016/j.vetmic.2008.11.010.

Miyama, T., Takanaga, H., Matsuo, H., Yamano, K., Yamamoto, K., Iga, T., Naito, M., Tsuruo, T., Ishizuka, H., Kawahara, Y., Sawada, Y., 1998. P-glycoprotein-mediated transport of itraconazole across the blood-brain barrier. Antimicrob. Agents Chemother. 42, 1738–1744.

Murphy, B.G., Perron, M., Murakami, E., Bauer, K., Park, Y., Eckstrand, C., Liepnieks, M., Pedersen, N.C., 2018. The nucleoside analog GS-441524 strongly inhibits feline infectious peritonitis (FIP) virus in tissue culture and experimental cat infection studies. Vet. Microbiol. 219, 226–233. https://doi.org/10.1016/j.vetmic.2018.04.026.

Palella, F.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J., Holmberg, S.D., 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N. Engl. J. Med. 338, 853–860. https://doi.org/10.1056/nejm199803263381301.

Pedersen, N.C., 2014. An update on feline infectious peritonitis: virology and immunopathogenesis. Vet. J. 201, 123–132. https://doi.org/10.1016/j.tvjl.2014.04.017.

Pedersen, N.C., Perron, M., Bannasch, M., Montgomery, E., Murakami, E., Liepnieks, M., Liu, H., 2019. Efficacy and safety of the nucleoside analog GS-441524 for treatment of cats with naturally occurring feline infectious peritonitis. J. Feline Med. Surg. 21, 271–281. https://doi.org/10.1177/1098612X19825701.

Poorolajal, J., Hooshmand, E., Mahjub, H., Esmailnasab, N., Jenabi, E., 2016. Survival rate of AIDS disease and mortality in HIV-infected patients: a meta-analysis. Public Health. https://doi.org/10.1016/j.puhe.2016.05.004.

Rodger, A.J., Cambiano, V., Bruun, T., Vernazza, P., Collins, S., Van Lunzen, J., Corbelli, G.M., Estrada, V., Geretti, A.M., Beloukas, A., Asboe, D., Viciana, P., Gutiérrez, F., Clotet, B., Pradier, C., Gerstoft, J., Weber, R., Westling, K., Wandeler, G., Prins, J.M., Rieger, A., Stoeckle, M., Kümmerle, T., Bini, T., Ammassari, A., Gilson, R., Krznaric, I., Ristola, M., Zangerle, R., Handberg, P., Antela, A., Allan, S., Phillips, A.N., Lundgren, J., 2016. Sexual activity without condoms and risk of HIV transmission in serodifferent couples when the HIV-positive partner is using suppressive antiretroviral therapy. JAMA 316, 171–181. https://doi.org/10.1001/jama.2016.5148.

Sun, D., 2020. Remdesivir for treatment of COVID-19: combination of pulmonary and IV administration may offer aditional benefit. AAPS J. 22, 77. https://doi.org/10.1208/s12248-020-00459-8.

Tai, D.Y.H., 2007. Pharmacologic Treatment of SARS: Current Knowledge and Recommendations. Ann. Acad. Med, Singapore.

Takano, T., Endoh, M., Fukatsu, H., Sakurada, H., Doki, T., Hohdatsu, T., 2017. The cholesterol transport inhibitor U18666A inhibits type I feline coronavirus infection. Antivir. Res. 145, 96–102. https://doi.org/10.1016/j.antiviral.2017.07.022.

Takano, T., Akiyama, M., Doki, T., Hohdatsu, T., 2019a. Antiviral activity of itraconazole against type i feline coronavirus infection. Vet. Res. 50 https://doi.org/10.1186/s13567-019-0625-3.

Takano, T., Wakayama, Y., Doki, T., 2019b. Endocytic pathway of feline coronavirus for cell entry: differences in serotype-dependent viral entry pathway. Pathogens 8. https://doi.org/10.3390/pathogens8040300.

Tekes, G., Thiel, H.J., 2016. Feline coronaviruses: pathogenesis of feline infectious peritonitis. In: Advances in Virus Research. Academic Press, pp. 193–218. https://doi.org/10.1016/bs.aivir.2016.08.002.

Xie, J., Wang, Z., 2021. Can remdesivir and its parent nucleoside GS-441524 be potential oral drugs? An in vitro and in vivo DMPK assessment. Acta Pharm. Sin. B 11, 1607–1616.

Yan, V.C., Pham, C.D., Yan, M.J., Yan, A.J., Khadka, S., Arthur, K., Ackroyd, J.J., Georgiou, D.K., Roon, L.E., Bushman, L.R., Anderson, P.L., Li, C., Muller, F.L., 2021. Pharmacokinetics of orally administered GS-441524 in dogs. bioRxiv. https://doi.org/10.1101/2021.02.04.429674. Preprint.