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Short Communication

Antiviral effect of mefloquine on feline calicivirus *in vitro*Phillip McDonagh^a, Paul A. Sheehy^{b,1}, Anne Fawcett^{c,2}, Jacqueline M. Norris^{a,*}^a Faculty of Veterinary Science, Building B14, The University of Sydney, Sydney, NSW 2006, Australia^b Faculty of Veterinary Science, Building B19, The University of Sydney, Sydney, NSW 2006, Australia^c Faculty of Veterinary Science, Building B03, The University of Sydney, Sydney, NSW 2006, Australia

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ABSTRACT

Feline calicivirus (FCV) is an important viral pathogen of domestic cats causing clinical signs ranging from mild to severe oral ulceration or upper respiratory tract disease through to a severe fatal systemic disease. Current therapeutic options are limited, with no direct acting antivirals available for treatment. This study screened a panel of 19 compounds for potential antiviral activity against FCV strain F9 and recent field isolates *in vitro*. Using a resazurin-based cytopathic effect (CPE) inhibition assay, mefloquine demonstrated a marked inhibitory effect on FCV induced CPE, albeit with a relatively low selectivity index. Orthogonal assays confirmed inhibition of CPE was associated with a significant reduction in viral replication. Mefloquine exhibited a strong inhibitory effect against a panel of seven recent FCV isolates from Australia, with calculated IC₅₀ values for the field isolates approximately 50% lower than against the reference strain FCV F9. *In vitro* combination therapy with recombinant feline interferon- ω , a biological response modifier currently registered for the treatment of FCV, demonstrated additive effects with a concurrent reduction in the IC₅₀ of mefloquine. These results are the first report of antiviral effects of mefloquine against a calicivirus and support further *in vitro* and *in vivo* evaluation of this compound as an antiviral therapeutic for FCV.

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1. Introduction

In domestic cats, feline calicivirus (FCV) is a common and important pathogen (Radford et al., 2007) typically resulting in upper respiratory tract signs or ulcerative oral lesions. Less frequent disease manifestations include a lameness syndrome, pneumonia, feline chronic gingivostomatitis syndrome (FCGS) (Lyon, 2005) and more recently the recognition of FCV-associated virulent systemic

disease (FCV-VSD), a condition associated with high morbidity and mortality, even in vaccinated adult cats (Hurley and Sykes, 2003; Pedersen et al., 2000).

Both inactivated and modified live FCV vaccines are available to help control calicivirus-related disease in cats. They are generally effective at reducing the severity and duration of clinical signs, but do not prevent infection or shedding (Radford et al., 2007). The high level of antigenic variability amongst FCV isolates has raised concerns regarding the level of cross protection afforded by older vaccines (Radford et al., 2006), and the apparent limited protection afforded against isolates associated with FCV-VSD (Hurley and Sykes, 2003). Given these concerns and the significant impact of the virus in some cats, a safe and effective antiviral therapeutic would significantly advance feline medicine.

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There are currently no direct acting antiviral drugs registered for the treatment of FCV, although the immune modulating drug recombinant feline interferon- ω (rFeIFN- ω), which likely has indirect antiviral properties, has a registered indication for FCV treatment. Efficacy of both human and feline interferons has been demonstrated *in vitro* against FCV (Fulton and Burge, 1985; Mochizuki et al., 1994; Taira et al., 2005; Truyen et al., 2002) and the use of rFeIFN- ω has been reported to have a positive therapeutic effect in FCV infected cats in experimental and field efficacy trials (Ninomiya et al., 1991; Ohe et al., 2008). Treatment with rFeIFN- ω was also associated with an improvement in clinical signs in cats with refractory FCGS in a double-blinded placebo-controlled study, however FCV shedding was not monitored, making it unclear whether the improvement was due to the antiviral or immunomodulatory properties of interferon (Hennet et al., 2011). Recently, feline calicivirus specific antiviral phosphorodiamidate morpholino oligomers (PMO) were tested in naturally occurring outbreaks of FCV-VSD (Smith et al., 2008) and were highly efficacious, with treatment resulting in improved survival, reduction in shedding, and a more rapid clinical recovery.

The current study screened a panel of compounds for antiviral activity against FCV using a resazurin-based CPE inhibition assay. The antiviral effects of compounds identified during screening were confirmed with plaque reduction and virus yield reduction assays. Effective compounds were tested against a panel of recent FCV field isolates from Australia to confirm their effectiveness against more clinically relevant viruses. Effective compounds were also tested in combination with rFeIFN- ω , currently the only licenced treatment for FCV in Australia.

2. Materials and methods

2.1. Cell culture and viruses

Crandell Rees feline kidney (CRFK) cell line was propagated as outlined previously (McDonagh et al., 2014). The reference feline calicivirus strain F9 was kindly provided by Professor Gilkerson (University of Melbourne). Field isolates of FCV were collected from cats in Sydney and Melbourne and grown on CRFK cells. To confirm FCV as the causative agent of CPE from clinical samples, an indirect immunofluorescence assay was performed using an anti-FCV monoclonal antibody (clone S1-9; Custom Monoclonals International, Sacramento, USA). All viruses were titrated by a carboxymethylcellulose plaque assay on CRFK cells and stored in single use aliquots at -80°C .

2.2. Test compounds

Nineteen compounds were selected based on reported *in vitro* antiviral effects against other RNA viruses (McDonagh et al., 2014). To determine an appropriate screening concentration the cytotoxicity of compounds was investigated using sequential resazurin and sulforhodamine B assays as previously reported (McDonagh et al., 2014). Test compound concentrations selected for subsequent antiviral screening were those resulting in cell

viability of 80% or greater and are shown in Table 1. Stock solutions were prepared as outlined previously (McDonagh et al., 2014). The maximum final in-well DMSO concentration used in these studies was 0.33%, which was demonstrated to have no significant effect on cell viability or viral replication (data not shown).

2.3. Antiviral screening using CPE inhibition assay

Antiviral screening was performed using a modification of the optimised resazurin-based CPE inhibition assay outlined previously (McDonagh et al., 2014). After 1 h of compound exposure cells were infected with FCV strain F9 at MOI 0.01 ($20\ \mu\text{l well}^{-1}$) for an infection period of 48 h with $50\ \mu\text{l}$ of 1:10 dilution of $4 \times$ stock resazurin in DMEM (final well concentration of resazurin 44 nM) added for the final 3.5 h. The duration of compound exposure in this assay was from 1 h prior to infection through to the assay endpoint, thereby allowing the identification of agents acting at any stage of the viral lifecycle. Plates were removed from the incubator for the final 30 min to allow the plates and media to equilibrate to room temperature. Fluorescent signals were measured with a FLUOstar Omega microplate reader (BMG Labtech, Mornington, VIC, Australia) using a 544 nm excitation filter and 590 nm emission filter with 8 flashes per well in bottom reading mode. Each treatment was performed in triplicate and results represent Mean \pm SE of three independent experiments. The percentage inhibition of CPE was calculated by:

$$\text{CPE inhibition(\%)} = \frac{\text{RFU}_{\text{Tx}} - \text{RFU}_{\text{V}(+)}}{\text{RFU}_{\text{V}(-)} - \text{RFU}_{\text{V}(+)}} \times 100$$

where RFU_{Tx} is the mean fluorescence intensity of treated infected cells; $\text{RFU}_{\text{V}(+)}$ is the mean fluorescence intensity in untreated infected cells; and $\text{RFU}_{\text{V}(-)}$ is the mean

Table 1
Compounds selected for antiviral screening.

Compound	Screening concentration
Chloroquine diphosphate ^a	25 μM
Quercetin ^a	10 μM
Curcumin ^b	10 μM
Rutin trihydrate ^b	25 μM
Indomethacin ^b	10 μM
Glycyrrhizic acid ^a	25 μM
Hesperidin ^b	50 μM
Aurintricarboxylic acid ^a	2.5 μM
Hesperetin ^b	50 μM
Mefloquine hydrochloride ^a	10 μM
Artesunate ^a	1 μM
Ribavirin ^a	2.5 μM
Baicalin hydrate ^b	10 μM
Hexamethylene amiloride ^b	10 μM
Cinanserin ^b	20 μM
Artemisinin ^a	25 μM
Niclosamide ^b	0.25 μM
Lactoferrin ^a	0.5 mg ml^{-1}
Recombinant feline interferon ω^c	100 units ml^{-1}

Superscripts indicate compound supplier.

^a Sigma–Aldrich.

^b Santa Cruz Biotechnology.

^c Virbac.

fluorescence intensity of untreated uninfected cells. Compounds showing marked, moderate, or mild antiviral effects were defined as those showing 75–100%, 50–74%, and 25–49% inhibition of CPE respectively. Compounds demonstrating marked CPE inhibition were classified as candidate compounds and were selected for further characterisation.

2.4. Titration of effective compounds and determination of selectivity index

Using the optimised FCV CPE inhibition assay, a concentration–response experiment was conducted with serial dilutions of effective compounds identified during screening. To enable calculation of the selectivity index a repeat cytotoxicity screen was performed concurrently. The cytotoxicity screen was performed as per the CPE inhibition assay with the exception that cells were mock infected with DMEM. Each treatment was performed in triplicate and repeated in three independent experiments, with results presented as Mean \pm SE. Data analysis were conducted in GraphPad Prism (V5.03 for Windows, GraphPad Software, San Diego, CA, USA), with the 50% inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) values calculated using the inbuilt non-linear curve fitting functions following \log_{10} transformation of compound concentrations. The selectivity index (SI) for each compound was calculated according to the following formula:

$$SI = \frac{CC50}{IC50}$$

2.5. Confirmatory assays

Plaque reduction and virus yield reduction assays were performed to confirm antiviral effects of candidate compounds identified using a modification of the methods previously described (McDonagh et al., 2014). For the virus yield reduction assay five concentrations of test compound were assessed. Cells were exposed to compounds for 1 h prior to infection with FCV F9 at MOI of 0.05 in 25 μ l DMEM. At 12 and 24 h post infection (hpi) cellular morphology was assessed for CPE using an inverted phase contrast microscope (CKX41, Olympus) and supernatant was harvested and stored at -80°C prior to titration of

extracellular virus using the TCID50 assay. The relative viral titre was calculated for each treatment with the value of the untreated control defined as 100%. Each treatment and time point was performed in triplicate and repeated in two independent experiments. Results represent Mean \pm SE. Plaque reduction assays were performed using a modification of the plaque assay previously described (McDonagh et al., 2014). Approximately 60 pfu FCV F9 was added per well. The relative plaque number was calculated for each treatment with the value of untreated control defined as 100%. Each treatment was performed in duplicate, and the results represent Mean \pm SE of three independent experiments.

2.6. Antiviral efficacy against field isolates of FCV

Using the resazurin-based-CPE inhibition assay, the antiviral efficacy of identified candidate compounds against seven currently circulating field isolates was investigated. Details of the field isolates are shown in Table 2. Assay conditions were identical to those used for screening except that cells were infected with different field isolates. Untreated cells were also infected with the different isolates as virus controls and uninfected and untreated cells included as positive controls. Each treatment was performed in triplicate wells for each virus, and repeated in three independent experiments. Data expressed as Mean \pm SE. To further investigate the antiviral efficacy against different isolates a resazurin-based CPE inhibition concentration–response study was conducted using field isolates 178N and IW1E. Each treatment was performed in triplicate and results represent Mean \pm SE of three independent experiments. Calculation of IC50, CC50, and SI values was performed as previously described.

2.7. Combination treatment with mefloquine and rFelfN- ω

Using the optimised resazurin-based CPE inhibition assay, cells were pre-treated with varying combinations of rFelfN- ω and mefloquine at concentrations from 0 to 1000 units ml^{-1} and 0 to 12 μM respectively prior to infection with FCV F9 at MOI 0.01. Each treatment was performed in triplicate and repeated in three independent experiments with data presented as Mean \pm SE. IC50 values were calculated as previously described.

Table 2

Details of FCV isolates used in experimental studies.

Strain	Disease manifestation/site of isolation	Location of isolation	Passage
F9	Vaccine strain	USA	P2 ^a
83E	Conjunctival swab from cat with oro-respiratory disease	Melbourne, Australia	P3
131M	Oropharyngeal swab from cat with oro-respiratory disease	Melbourne, Australia	P3
178N	Nasal swab from cat with oro-respiratory disease	Melbourne, Australia	P3
IW1E	Pharyngeal swab from cat with FCGS	Sydney, Australia	P2
IW10	Pharyngeal swab from cat with oro-respiratory disease	Sydney, Australia	P2
IW16	Swab from tongue ulcer noted as incidental finding during pre-general anaesthetic examination	Sydney, Australia	P2
IW25	Oropharyngeal swab from a cat with stomatitis	Sydney, Australia	P2

^a Passage 2 within our laboratory, as original passage number unknown.

3. Results

3.1. Identification of mefloquine as an inhibitor of FCV replication *in vitro*

Mefloquine, at a concentration of 10 μM , was the only compound of the 19 tested that demonstrated marked inhibition (88.6%) of virus induced CPE (Fig. 1). All other compounds demonstrated a limited, or no inhibitory effect on CPE. Included among these are four compounds – ribavirin, lactoferrin, chloroquine, and rFeIFN- ω – that had previously demonstrated *in vitro* efficacy against FCV (Kreutz and Seal, 1995; McCann et al., 2003; Povey, 1978; Truyen et al., 2002). Titration of mefloquine demonstrated a clear concentration–response relationship with almost complete inhibition of CPE at the highest tested concentrations reducing to zero inhibition at lowest tested concentration. Calculated IC₅₀, CC₅₀, and SI values are shown in Fig. 2.

Orthogonal testing confirmed CPE inhibition was associated with marked reductions in the production of progeny virions. Using a virus yield reduction assay mefloquine demonstrated a concentration dependent inhibition of viral replication when assayed at 12 and 24 hpi (Fig. 3). At high concentrations, reductions in viral titre at 24 hpi was greater than 3 log₁₀, while at 12 hpi this was reduced to between 1 and 2 log₁₀. Calculated IC₅₀ and SI values at 12 and 24 hpi are shown in Fig. 3. Plaque reduction assays confirmed the findings of the resazurin-based CPE inhibition assay. Pre-treatment with mefloquine resulted in a concentration-dependent reduction in plaque

number, with a calculated IC₅₀ of 6.03 μM (95% confidence interval 5.43–6.70) and resulting selectivity index of 3.7. Plaque size and morphology varied considerably in both treated and untreated cells, however in general plaque size was smaller and plaque morphology more consistent in wells treated with higher concentrations of mefloquine.

3.2. Antiviral efficacy against field isolates

Mefloquine demonstrated similar inhibition of virus induced CPE against all tested field isolates to that of the reference strain FCV F9 (Fig. 4). To assess any quantitative difference in the efficacy of mefloquine against different field isolates, a concentration–response experiment was conducted using field isolates IW1E and 178N. Mefloquine demonstrated concentration-dependent inhibition of both isolates, with similar shaped concentration–response curves and IC₅₀ values less than half that of FCV F9 (Fig. 5).

3.3. Effect of combination treatment with mefloquine and rFeIFN- ω

rFeIFN- ω exerted concentration-dependent additive antiviral effects *in vitro* when combined with mefloquine (Fig. 6). Increasing the rFeIFN- ω concentration from 0 to 500 units ml⁻¹ resulted in a shift of the concentration–response curve to the left with a corresponding decrease in the calculated IC₅₀ value. With this assay the additive effect of rFeIFN- ω on the antiviral efficacy of mefloquine

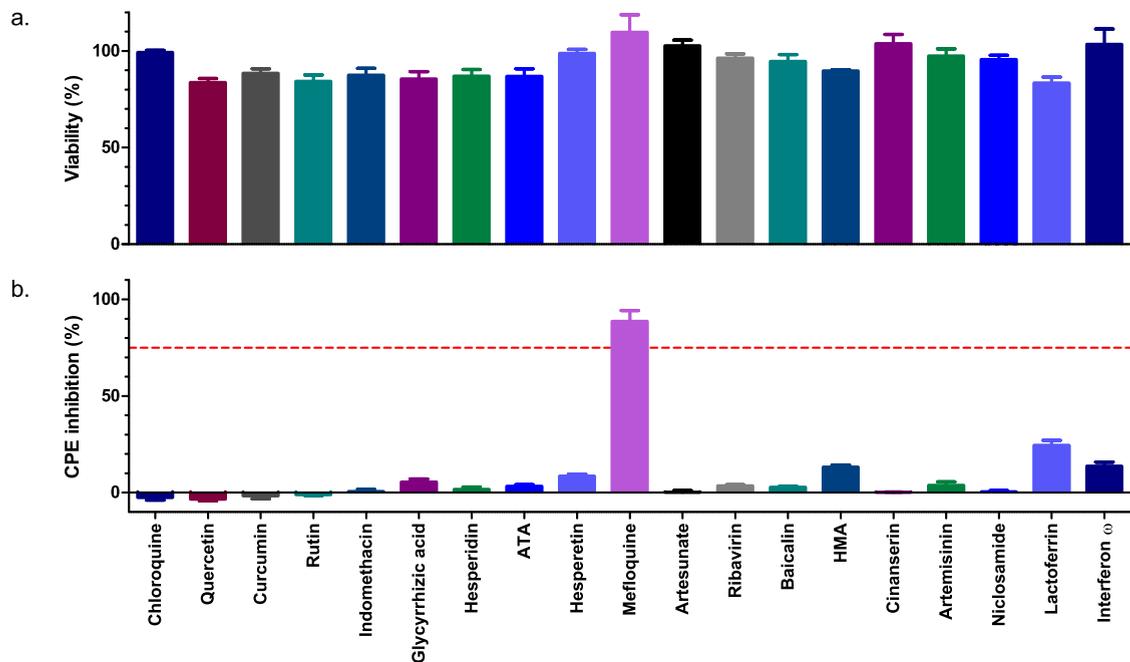


Fig. 1. Results of FCV antiviral screening experiment. Cells were pre-treated with various compounds for 1 h prior to infection with FCV F9 at MOI 0.01. Antiviral efficacy was determined 48 hpi using the resazurin-based CPE inhibition assay (b). A concurrent cytotoxicity screen was performed using the same protocol with the exception that cells were mock infected (a). Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean \pm SE. ATA, aurointricarboxylic acid; HMA, hexamethylene amiloride. Red dotted line = 75% inhibition of CPE.

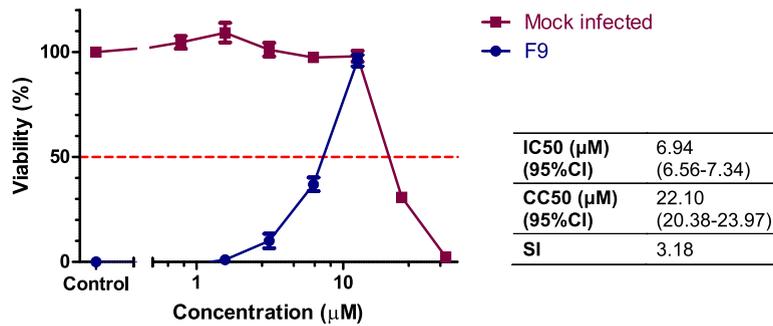


Fig. 2. Titration of mefloquine against FCV using the resazurin-based CPE inhibition assay. Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean \pm SE. Calculated IC₅₀, CC₅₀, and SI values are shown in the accompanying table.

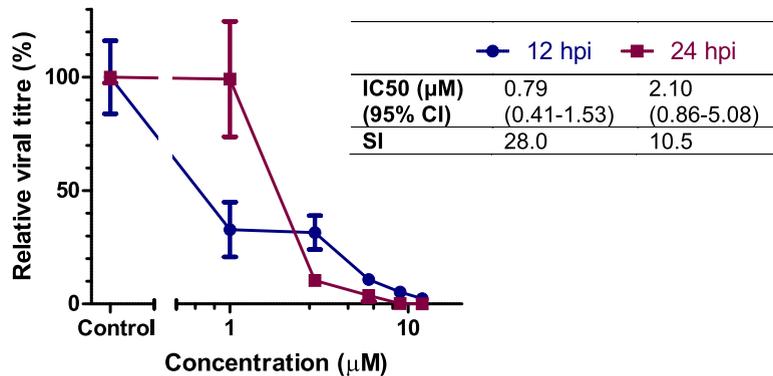


Fig. 3. Virus yield reduction assay for mefloquine against FCV. Cells were pre-treated with various dilutions of mefloquine for 1 h prior to infection with FCV F9 at MOI 0.05. Extracellular virus titre was calculated at 12 (blue circles) and 24 hpi (red squares) with a TCID₅₀ end point assay. Titre of untreated control is defined as 100%. Each treatment was performed in triplicate and repeated in two independent experiments. Data represent Mean \pm SE. Calculated IC₅₀ and SI values for each time point are shown in the accompanying table.

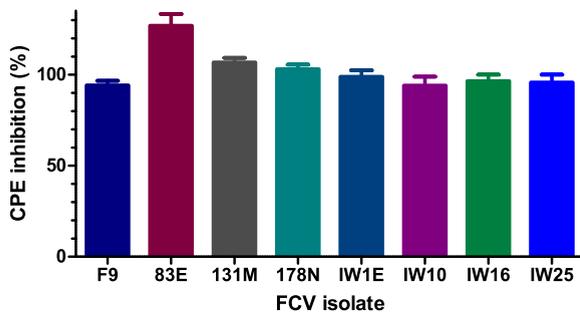


Fig. 4. Antiviral efficacy of mefloquine against recent Australian field isolates of FCV. Antiviral efficacy was determined 48 hpi using the resazurin-based CPE inhibition assay. Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean \pm SE.

appeared to peak at 500 units ml⁻¹ with greater concentrations not resulting in further reductions in the IC₅₀ value.

4. Discussion

We have demonstrated a marked inhibitory effect of mefloquine on the replication and associated cytopathic

effects of FCV in cell culture. Initial screening and confirmatory assays were performed with the culture adapted vaccine strain F9; however subsequent testing against seven current Australian field isolates demonstrated excellent antiviral efficacy remained potentially more relevant viruses, with concentration–response studies confirming that mefloquine is a more potent inhibitor of field isolates than the reference strain. Combination treatment with rFeIFN- ω resulted in additive effects with a reduction in the IC₅₀ value for mefloquine. These data provide important preliminary information on the potential use of mefloquine or its derivatives as an antiviral therapeutic for the treatment of FCV-associated diseases.

Initial screening and the orthogonal confirmatory assays were performed with FCV F9, a well-characterised strain originally isolated in 1958 (Glenn et al., 1999). FCV F9 was chosen for screening due to its well characterised *in vitro* growth properties, however it is unclear whether FCV F9 used in the current study is representative of currently circulating viruses, due to natural viral evolution of circulating field viruses over the last half century in face of vaccination with F9 based vaccines, or cell culture adaptation, as can occur with high passage number. It is therefore possible that results obtained against FCV F9 may not be representative of efficacy against circulating strains,

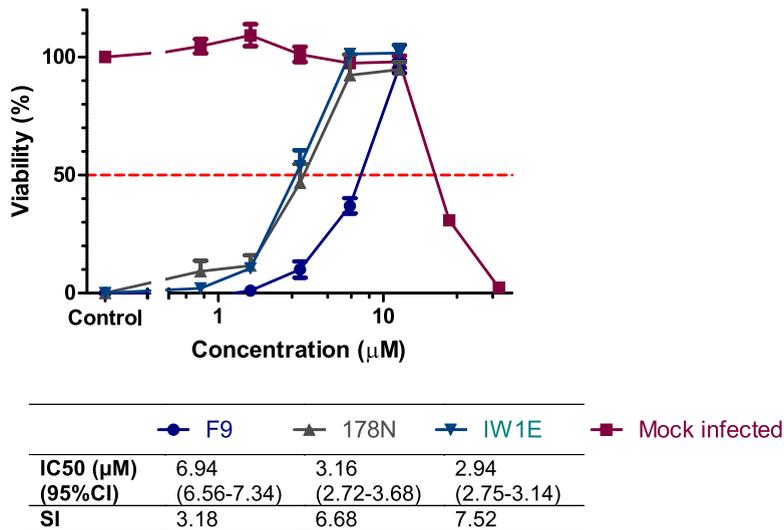


Fig. 5. Antiviral titration of mefloquine against Australian field isolates of FCV. Antiviral efficacy was determined 48 hpi using the resazurin-based CPE inhibition assay. Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean ± SE.

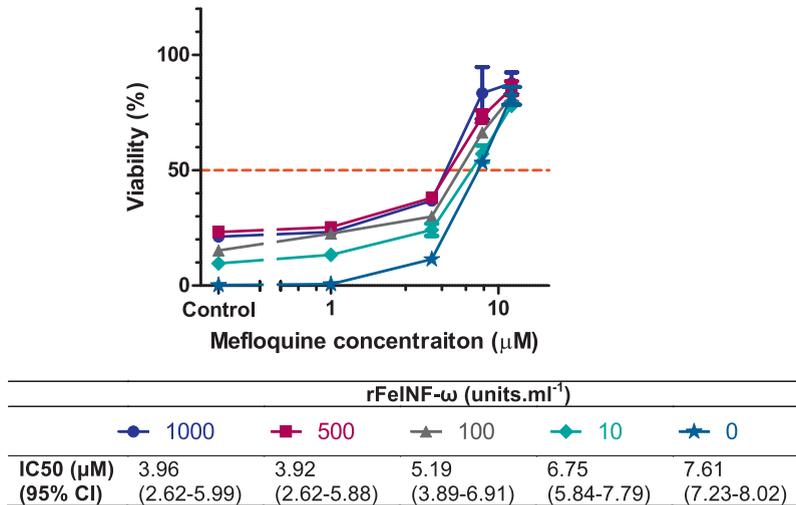


Fig. 6. Effect of combination treatment with mefloquine and rFeINF-ω against FCV F9. Cells were pre-treated with varying combinations of rFeINF-ω and mefloquine prior to infection with FCV F9 at MOI 0.01. Antiviral efficacy was determined 48 hpi using the resazurin-based CPE inhibition assay. Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean ± SE. IC50 values for mefloquine were calculated for each rFeINF-ω concentration.

particularly given the known genetic heterogeneity of FCV (Coyne et al., 2012). Additional antiviral screening was performed against a panel of seven recent FCV isolates from Australia. Variation in susceptibility to antiviral agents can arise rapidly due to viral evolution in response to selection pressures imposed by the use of an antiviral drug in a population, however it may also pre-exist in treatment naive populations. Ferraris et al. (2005) showed significant variability in the sensitivity of influenza A viruses to the neuraminidase inhibitors zanamivir and oseltamivir, even in viruses isolated prior to the widespread use of these drugs. In contrast, in the current study all tested FCV isolates were susceptible to the inhibitory effects of mefloquine based on the CPE inhibition assay. A concentration–response study demonstrated a greater

than two-fold increase in potency for the two field isolates tested compared to the reference virus FCV F9. Although further studies are required to determine whether this is a general feature of field isolates, or whether unique to the two tested isolates, it highlights that consideration must be given to the viral strain used for screening.

Combination antiviral therapy is common practice in significant human viral infections such as HIV and HCV (Feld and Hoofnagle, 2005; Shafer and Vuitton, 1999) and offers several potential theoretical benefits over monotherapy including a more rapid and complete inhibition of replication, a reduction in the likelihood of viral escape, and the potential to reduce the dose of individual drugs, and thereby minimise dose-dependent adverse effects. For HCV, combination therapy of ribavirin and interferon has

historically been the standard of care, and demonstrated good clinical success (Feld and Hoofnagle, 2005). As rFeIFN- ω is an approved therapeutic in cats we sought to investigate its use in combination with mefloquine. Interferon monotherapy has previously demonstrated small antiviral effects against FCV *in vitro* (Mochizuki et al., 1994; Truyen et al., 2002). *In vivo* efficacy has also been reported in experimental and field studies of FCV (Ninomiya et al. (1991), Uchino et al. (1991) cited in Ohe et al. (2008)). At the concentration used in the current study however, monotherapy with rFeIFN- ω provided limited protection from CPE which may reflect differences in the sensitivity of virus yield reduction assays compared to CPE inhibition assays (McDonagh et al., 2014) as well as differences in the experimental and infection conditions. Combination therapy with rFeIFN- ω and mefloquine however demonstrated additive effects, with increasing concentrations of interferon resulting in a reduction in the IC50 of mefloquine. This combination may prove a useful therapeutic option for FCV infections, particularly if it reduces the dose of mefloquine, and a possible concomitant reduction in the risk of adverse reactions.

The results of this study further expand the known antiviral spectrum of mefloquine with *in vitro* efficacy previously reported against HIV, JC virus, and feline coronavirus (Brickelmaier et al., 2009; McDonagh et al., 2014; Owen et al., 2005). The mechanism of action of mefloquine against this diverse group of viruses is not known, nor is it clear whether antiviral efficacy is due to a common broad spectrum mechanism of action. Investigations into the likely mechanism of action of mefloquine against FCV were not conducted in the current study. Given the documented effects of mefloquine on endocytosis, and the morphological changes suggestive of alterations in these pathways in CRFK cells (McDonagh et al., 2014) it is tempting to speculate that the antiviral effects against FCV may arise through perturbation of these pathways. Cellular entry of FCV occurs *via* receptor mediated endocytosis following binding to the cellular receptor fJAM-A (Stuart and Brown, 2006). Treatment with known inhibitors of different steps of viral entry, including inhibitors of endocytosis, revealed FCV entry involves clathrin coated pits and that endosome acidification was required for infection, in agreement with a previous report by Kreutz and Seal (1995) that showed that chloroquine inhibited viral replication. Under the assay conditions of the current study chloroquine provided no protection against virus induced CPE, suggesting that if viral inhibition was arising through the lysosomotropic effects of mefloquine (Glaumann et al., 1992), this effect is considerably more potent for mefloquine than for chloroquine. Alternatively, a mechanism unrelated to its accumulation in lysosomes, such as an action as an adenosine analogue as hypothesised for JC virus (Brickelmaier et al., 2009), may account for the observed antiviral effects.

Although mefloquine is a human approved pharmaceutical with a significant body of literature regarding its pharmacokinetics and safety, its use in cats has not been reported. It is well recognised that due to deficiencies in a

number of drug metabolism pathways, the pharmacokinetics of certain drugs in cats can be quite different to those of other species, a fact which should be considered when extrapolating human pharmacokinetic data to this species (Court, 2013). The IC50 value calculated for the field isolates, based on inhibition of CPE, was approximately 3 μ M which is lower than plasma concentrations of mefloquine reported in human studies (4–23 μ M depending on dosing regimens) (Kollaritsch et al., 2000; Simpson et al., 1999). Extrapolating from available data therefore, it may be possible to achieve *in vivo* concentrations of mefloquine within the therapeutic range; however the potential for cytotoxicity must be considered given the low SI of the compound in CRFK cells.

5. Conclusion

This study identified mefloquine as a potent inhibitor of FCV *in vitro* when present at low micromolar concentrations. This is the first report of the antiviral activity of mefloquine against a calicivirus. Testing against a panel of recent Australian FCV isolates demonstrated the antiviral effects of mefloquine against the reference strain FCV F9 extends to more clinically relevant isolates, and there was no evidence of antagonism when used concurrently with rFeIFN- ω . Further investigation and optimisation of this compound for clinical use in treating the more serious manifestations of FCV infection is warranted. Consideration should be given to investigating the effectiveness of mefloquine against other members of the *Caliciviridae* of medical or veterinary importance.

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