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# Identification and characterisation of small molecule inhibitors of feline coronavirus replication



Phillip McDonagh<sup>a</sup>, Paul A Sheehy<sup>b</sup>, Jacqueline M Norris<sup>a,\*</sup>

<sup>a</sup> Faculty of Veterinary Science, Building B14, The University of Sydney, Sydney, NSW 2006, Australia

<sup>b</sup> Faculty of Veterinary Science, Building B19, The University of Sydney, Sydney, NSW 2006, Australia

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## ABSTRACT

Feline infectious peritonitis (FIP), a feline coronavirus (FCoV) induced disease, is almost invariably fatal with median life expectancy measured in days. Current treatment options are, at best, palliative. The objectives of this study were to evaluate a panel of nineteen candidate compounds for antiviral activity against FCoV *in vitro* to determine viable candidates for therapy. A resazurin-based cytopathic effect inhibition assay, which detects viable cells through their reduction of the substrate resazurin to fluorescent resorufin, was developed for screening compounds for antiviral efficacy against FCoV. Plaque reduction and virus yield reduction assays were performed to confirm antiviral effects of candidate compounds identified during screening, and the possible antiviral mechanisms of action of these compounds were investigated using virucidal suspension assays and CPE inhibition and IFA-based time of addition assays. Three compounds, chloroquine, mefloquine, and hexamethylene amiloride demonstrated marked inhibition of virus induced CPE at low micromolar concentrations. Orthogonal assays confirmed inhibition of CPE was associated with significant reductions in viral replication. Selectivity indices calculated based on *in vitro* cytotoxicity screening and reductions in extracellular viral titre were 217, 24, and 20 for chloroquine, mefloquine, and hexamethylene amiloride respectively. Preliminary experiments performed to inform the antiviral mechanism of the compounds demonstrated all three acted at an early stage of viral replication. These results suggest that these direct acting antiviral compounds, or their derivatives, warrant further investigation for clinical use in cats with FIP.

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## 1. Introduction

Current treatment options for cats with the invariably fatal feline coronavirus (FCoV) induced disease, feline infectious peritonitis (FIP) are limited and palliative, with a median life expectancy typically measured in days or

weeks. The pathology of FIP is immune mediated, however the triggering and perpetuating event is the increased replication of FCoV in cells of the monocyte lineage (Pedersen, 2009), suggesting a therapeutic role for anti-FCoV agents in the treatment of this condition.

Treatment for FIP has mainly focused on immune modulating drugs. A limited number of studies purported successful treatments for FIP using immunomodulatory therapy, however larger, well controlled studies have not found the same positive outcomes (Fischer et al., 2011; Hartmann and Ritz, 2008; Ritz et al., 2007). Less has been reported on the use of direct acting antivirals against FCoV.

\* Corresponding author. Tel.: +61 2 9351 7095.

E-mail addresses: [phillip.mcdonagh@sydney.edu.au](mailto:phillip.mcdonagh@sydney.edu.au) (P. McDonagh), [paul.sheehy@sydney.edu.au](mailto:paul.sheehy@sydney.edu.au) (P.A. Sheehy), [jacqui.norris@sydney.edu.au](mailto:jacqui.norris@sydney.edu.au) (J.M. Norris).

A number of compounds have demonstrated an inhibitory effect on the virus *in vitro* (Barlough and Shacklett, 1994; Hsieh et al., 2010; Keyaerts et al., 2007), but there is little or no published data regarding their use in treating FIP. The broad spectrum antiviral ribavirin demonstrated *in vitro* efficacy but provided limited clinical benefit and produced toxicity in cats (Weiss et al., 1993). More recently in a small study involving experimentally infected cats treatment with chloroquine, a drug with demonstrated *in vitro* antiviral efficacy, was associated with mild improvements in clinical signs, however there was no statistically significant difference in survival time compared to untreated cats (Takano et al., 2013). Efficacious and safe antiviral therapeutics are desperately needed for FIP treatment.

Modern antiviral drug discovery often involves high throughput screening of vast chemical libraries. These large scale unfocused screens are expensive and beyond the reach of companion animal medicine. An alternative approach is to utilise a more focused screening strategy, enriching the screening library with compounds considered likely to have an antiviral effect based on a prior knowledge of their pharmacodynamics and the viral life cycle. Focused screening panels may consist of compounds related to those demonstrated effective against the challenge virus or those demonstrated effective against closely related viruses.

In the current study we screened 19 compounds with previously demonstrated antiviral activity against coronaviruses or other RNA viruses, for antiviral activity against FCoV using an optimised resazurin-based CPE inhibition assay. Cytotoxicity of compounds was determined prior to screening using sequential resazurin- and SRB-based assays to determine the optimal minimally toxic test concentration and to enable calculation of selectivity indices. The antiviral effects of compounds identified during screening were confirmed with plaque reduction and virus yield reduction assays. Virucidal suspension assays and time of addition assays provided initial information on the stage of viral replication targeted and the potential mechanism of action.

## 2. Materials and methods

### 2.1. Cell culture and viruses

Crandell Rees Feline Kidney (CRFK) cell line was propagated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Castle Hill, NSW, Australia) supplemented with 10% FBS (Sigma–Aldrich) (DMEM-10) in a humidified incubator at 37 °C in 5% CO<sub>2</sub> in air. Two strains of FCoV, FIPV WSU 79-1146 (FIPV1146) and FECV WSU 79-1683 (FECV1683), acquired from the American Type Culture Collection (Virginia, USA), were used. FCoV FECV1683 was originally isolated from mesenteric lymph nodes and intestinal washes of a 1.5 year old female domestic shorthaired cat that died of acute haemorrhagic gastroenteritis while FCoV FIPV1146 was originally isolated from the liver, spleen, and lungs from a case of neonatal death in a 4-day-old male Persian kitten (McKeirnan et al., 1981). Pathogenicity studies of these

two isolates have shown that FIPV1146 is highly virulent and reliably causes signs of classic FIP following oronasal inoculation, while FECV1683 causes a low grade fever and mild enteritis, but no signs of FIP (Pedersen, 2009). Despite the dissimilar *in vivo* biological properties of the two isolates, the two have similar *in vitro* properties in immortalised cell lines.

### 2.2. Test compounds

Compounds were selected for the test panel based on their reported *in vitro* antiviral properties against coronaviruses or other RNA viruses (see supplementary material for details). The compounds tested and their screening concentrations are shown in Table 1. Stock solutions were prepared by dissolving compounds in ultrapure water or DMSO (Sigma–Aldrich). Compounds were sterile filtered with a 0.22 µm regenerated cellulose filter (Corning Inc., Corning, NY, USA), aliquoted into sterile single use microtubes (Sarstedt, Numbrecht, Germany), and stored for a maximum of 6 months at –80 °C until use.

To determine an appropriate screening concentration, cytotoxicity of test compounds was determined using sequential resazurin and sulforhodamine B assays. The resazurin-based assay was performed as for the antiviral screening assay except compounds were added in 50 µl volume and there was no infection step. To perform the SRB assay, cells were immediately fixed post fluorescent data acquisition by decanting culture media by inverting plates and adding 10% trichloroacetic acid for 1 h at 4 °C. SRB staining was as previously described by (Vichai and Kirtikara, 2006) except that 0.2% SRB was used for staining. Following solubilisation of bound dye, OD510 was measured using the FLUOstar Omega microplate reader (BMG Labtech, Mornington, Australia). Viability was compared to untreated controls. Test compound concentrations selected for subsequent antiviral screening were those resulting in cell viability of 80% or greater.

**Table 1**

Compounds selected for antiviral screening. Superscripts indicate compound supplier: \*, Sigma–Aldrich; †, Santa Cruz Biotechnology; ‡, Virbac.

Compound	Screening concentration
Chloroquine diphosphate <sup>*</sup>	25 µM
Quercetin <sup>*</sup>	10 µM
Curcumin <sup>†</sup>	10 µM
Rutin trihydrate <sup>†</sup>	25 µM
Indomethacin <sup>†</sup>	10 µM
Glycyrrhizic acid <sup>*</sup>	25 µM
Hesperidin <sup>†</sup>	50 µM
Aurintricarboxylic acid <sup>*</sup>	2.5 µM
Hesperetin <sup>†</sup>	50 µM
Mefloquine hydrochloride <sup>*</sup>	10 µM
Artesunate <sup>*</sup>	1 µM
Ribavirin <sup>*</sup>	2.5 µM
Baicalin hydrate <sup>†</sup>	10 µM
Hexamethylene amiloride <sup>†</sup>	10 µM
Cinanserin <sup>†</sup>	20 µM
Artemisinin <sup>*</sup>	25 µM
Niclosamide <sup>†</sup>	0.25 µM
Lactoferrin <sup>*</sup>	0.5 mg ml <sup>-1</sup>
Recombinant feline interferon ω <sup>‡</sup>	100 units ml <sup>-1</sup>

### 2.3. Antiviral screening using CPE inhibition assay

Antiviral screening was performed using an optimised resazurin-based CPE inhibition assay. Clear-bottomed black-walled 96-well plates ( $\mu$ Clear®, Greiner Bio-One, Frickenhausen, Germany) were seeded with  $1.25 \times 10^4$  cells well<sup>-1</sup> in 100  $\mu$ l DMEM-10 (or 100  $\mu$ l DMEM-10 for control wells containing no cells). Plates were held at room temperature for 30 min post-seeding then incubated then at 37 °C in 5% CO<sub>2</sub> in air for 5 h prior to compound addition. Compounds were diluted in DMEM, and 30  $\mu$ l added per well. After 1 h of compound exposure cells were infected with FCoV FIPV1146 at MOI 0.01 (20  $\mu$ l well<sup>-1</sup>) for an infection period of 72 h with 50  $\mu$ l of 1:10 dilution of 4 $\times$  stock resazurin (Sarstedt) in DMEM (final well concentration of resazurin 44 nM) added for the final 3.5 h. Plates were removed from the incubator for the final 30 min to allow plates and media to equilibrate to room temperature. Fluorescent signals were measured with a FLUOstar Omega microplate reader 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:

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

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 resazurin-based CPE inhibition assay a concentration–response experiment was conducted with serial dilutions of identified candidate compounds (nine concentrations per compound). To enable calculation of the selectivity index, a repeat cytotoxicity screen was performed concurrently. Each treatment was performed in triplicate and repeated in three independent experiments. Data were exported to Microsoft Excel for calculation of cell viability and CPE inhibition according to the formulae described above. Data analysis were conducted in GraphPad Prism, with the 50% inhibitory concentration (IC<sub>50</sub>) and 50% cytotoxic concentration (CC<sub>50</sub>) values calculated using the inbuilt non-linear curve fitting functions following log<sub>10</sub> transformation of compound concentrations. The selectivity index (SI) for each compound was calculated according to the following formula:

$$\text{SI} = \frac{\text{CC}_{50}}{\text{IC}_{50}}$$

### 2.5. Confirmatory assays

Plaque reduction and virus yield reduction assays were performed to confirm antiviral effects of candidate compounds identified using the CPE inhibition assay. Virus yield reduction assays were performed in 24-well plates (Sarstedt). Wells were seeded with  $4.0 \times 10^4$  cells well<sup>-1</sup> in 400  $\mu$ l DMEM-10. Plates were kept at room temperature for 30 min and then at 37 °C in 5% CO<sub>2</sub> in air for 5 h prior to the addition of test compounds. Compounds were diluted in DMEM to the required concentrations with 75  $\mu$ l added to each well. Cells were incubated at 37 °C in 5% CO<sub>2</sub> in air for an additional 1 h prior to infection with FCoV FIPV1146 at MOI 0.1 in 25  $\mu$ l DMEM. Cells were incubated for a further 48 h at 37 °C in 5% CO<sub>2</sub>. At 24 and 48 h post-infection (hpi) cell monolayers were visually assessed for CPE using an Olympus CKX41 inverted phase-contrast microscope (Olympus, Melville, NY, USA) and culture media was collected and stored at –80 °C for virus titration. Untreated infected cells, untreated uninfected cells, and treated uninfected cells were included as controls. This latter control was included to allow assessment of morphological changes to cells due to compound treatment. Titration of extracellular virus harvested at 24 and 48 hpi was performed using the TCID<sub>50</sub> method as described by McDonagh et al. (2011). Each treatment and time point was performed in triplicate and repeated in two independent experiments, with results representing Mean  $\pm$  SE.

Plaque reduction assays were performed in 12-well plates (Corning). Cells seeded at a density of  $6 \times 10^4$  cells well<sup>-1</sup> in 1 ml DMEM-10 were held at room temperature for 30 min prior to incubation at 37 °C in 5% CO<sub>2</sub> in air for 60 h, by which time monolayers were approximately 90% confluent. Culture media was discarded and replaced with 400  $\mu$ l DMEM supplemented with 2% FBS plus 75  $\mu$ l of various concentrations of test compounds in DMEM (or 75  $\mu$ l DMEM only for control wells) using five or six concentrations per compound. After exposure to the compound for 1 h, cells were infected with 30 pfu well<sup>-1</sup> FCoV FIPV1146 in 25  $\mu$ l DMEM. Virus was allowed to adsorb for 90 min with plates rocked every 15 min to ensure an even distribution of inoculum. Culture media was discarded after 90 min and cells overlaid with 1 ml 0.9% carboxymethylcellulose, 2% FBS in DMEM containing the same concentration of compound as present prior to and during infection. Cells were fixed and stained with 0.1% (w/v) crystal violet 48 hpi prior to manual plaque counting. 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 repeated in three independent experiments, with data representing Mean  $\pm$  SE.

### 2.6. Virucidal suspension assay

A virucidal suspension assay was performed to assess virucidal effects of test compounds. The assay was performed as above with the exception that virus was mixed and incubated with test compounds prior to infection. Stock FCoV FIPV1146, diluted in DMEM to

$2 \times 10^6$  pfu ml<sup>-1</sup>, was mixed with an equal volume of test compound diluted in DMEM to  $2 \times$  the test concentration used during screening. The control virus suspension was mixed with DMEM containing an equal concentration of DMSO as the test samples. Virus suspensions were incubated for 1 h at room temperature before serial dilution in DMEM to infect cells with 25 pfu well<sup>-1</sup> in 100  $\mu$ l. Following serial dilution of the virus, cells were exposed to test compounds at concentrations greater than 4 log<sub>10</sub> lower than concentrations previously shown to have no antiviral effect. The experiment was performed in triplicate and repeated in two independent experiments. Data represent Mean  $\pm$  SE.

### 2.7. Time of addition assays

A modification of the resazurin-based CPE inhibition assay was performed to assess the effect of time of compound addition on the antiviral efficacy of identified compounds. The CPE inhibition assay was performed as previously described with the exception that test compounds were added at various time points before and after infection. The selected time points were 1 h prior to infection, concurrent with infection, and 1, 3, or 6 h post-infection. Treatments were performed in triplicate and repeated in three independent experiments. Data represent Mean  $\pm$  SE.

To further elucidate the stage of viral replication affected by each compound the effect of time of addition on viral antigen expression was examined. Cells were seeded at a density of  $5.0 \times 10^3$  cells well<sup>-1</sup> in 100  $\mu$ l DMEM-10 in 96-well plates ( $\mu$ Clear®, Greiner Bio-One). After seeding plates were kept at room temperature for 30 min and then incubated at 37 °C in 5% CO<sub>2</sub> in air for 5 h prior to the first time-point of compound addition. Compounds were added in 30  $\mu$ l to duplicate wells at different time points prior to, concurrent with, or post-infection. Cells were infected with FCoV FIPV1146 at MOI 0.5 in 20  $\mu$ l or mock infected with 20  $\mu$ l DMEM for an infection period of 1 h. An infection period of 12 h was selected based on the reported one step growth curve of FCoV (Rottier et al., 2005). At 12 hpi (measured from the end of the infection period) cells were fixed in 20% formaldehyde in PBS and permeabilised in ice cold methanol. Viral antigen was detected with a biotinylated anti-FCoV antibody (CCV2-2; Custom Monoclonals International, Sacramento, CA, USA) and visualised with streptavidin-conjugated Alexafluor 555 (Life Technologies, Mulgrave, VIC, Australia). To enable accurate segmentation, cells were stained with the whole cell stain HCS Cell Mask Blue (Life Technologies) and DAPI (Life Technologies) to enhance nuclear visualisation. Fluorescent imaging was performed using the BD Pathway 855 Bioimager (BD Bioscience, Franklin Lakes, NJ, USA). Images of wells were acquired using a 10 $\times$  objective (NA 0.4) using a 3  $\times$  3 montage with laser autofocus performed for each montage frame. HCS Cell Mask Blue/DAPI, images were acquired with Ex 380/10 BP and Em 435 LP filters, and Alexa Fluor 555 images acquired with Ex 548/20 BP and Em 570 LP filters. Image analysis was performed using the free open-source image analysis software CellProfiler (R11710,

[www.cellprofiler.org](http://www.cellprofiler.org)) with data exported to FCS Express Image Cytometry (version 4.07.0005, De Novo Software, Los Angeles, CA, USA) for analysis. Each treatment was performed in duplicate, and data represents Mean  $\pm$  SD.

### 2.8. Strain variation

To assess efficacy against different FCoV strains, identified candidate compounds were tested against FCoV FECV1683 using the resazurin-based CPE inhibition assay. The assay was performed as described, except that cells were infected with either FCoV FIPV1146 or FECV1683 at MOI 0.01. Each treatment was performed in triplicate and repeated in three independent experiments, with data representing Mean  $\pm$  SE.

## 3. Results

### 3.1. Identification of effective compounds

Three of nineteen tested compounds showed marked inhibition of virus induced CPE (Fig. 1) and were selected for further characterisation. Pre-treatment with chloroquine at 25  $\mu$ M, mefloquine at 10  $\mu$ M, and hexamethylene amiloride at 10  $\mu$ M resulted in 93.3%, 89.8%, and 77.6% inhibition of CPE respectively. A further two compounds, glycyrrhizic acid at 25  $\mu$ M and cinanserin at 20  $\mu$ M displayed a mild antiviral effect with a 26.7% and 34.0% reduction in CPE respectively. All other compounds demonstrated limited or no inhibitory effect on CPE. Included among these ineffective compounds was ribavirin, a broad spectrum antiviral compound that had previously shown *in vitro* (Barlough and Scott, 1990; Weiss and Oostrom-Ram, 1989), and to a limited extent *in vivo* efficacy against FCoV (Weiss et al., 1993), as well as rFelfN- $\omega$  which had previously shown *in vitro* efficacy against FCoV (Mochizuki et al., 1994; Truyen et al., 2002).

A concentration–response study was conducted with chloroquine, mefloquine, and hexamethylene amiloride. A repeat cytotoxicity screen was concurrently performed for these compounds to allow calculation of selectivity indices. All compounds demonstrated a clear concentration–response effect over the tested range (Fig. 2). Calculated IC<sub>50</sub>, CC<sub>50</sub>, and SI values for the compounds are shown in Table 2.

Virus yield reduction assays confirmed the CPE inhibition identified during screening was associated with a marked reduction in extracellular viral titre. Determination of extracellular virus titre was performed at 24 and 48 hpi with results shown in Fig. 3. For chloroquine and mefloquine there was a considerable difference in the resulting concentration–response curves at 24 and 48 hpi, while for hexamethylene amiloride the shape of the curve was similar at both time points. Differences in concentration–response curves between the two time points is reflected in the IC<sub>50</sub> values, with increased IC<sub>50</sub> values for chloroquine and mefloquine at 48 hpi compared to 24 hpi, while for hexamethylene amiloride IC<sub>50</sub> values were similar at both time points (Table 3). Plaque reduction assays confirmed the findings of the CPE inhibition and virus yield reduction assays. Pre-treatment with chloroquine, mefloquine, or hexamethylene amiloride resulted in

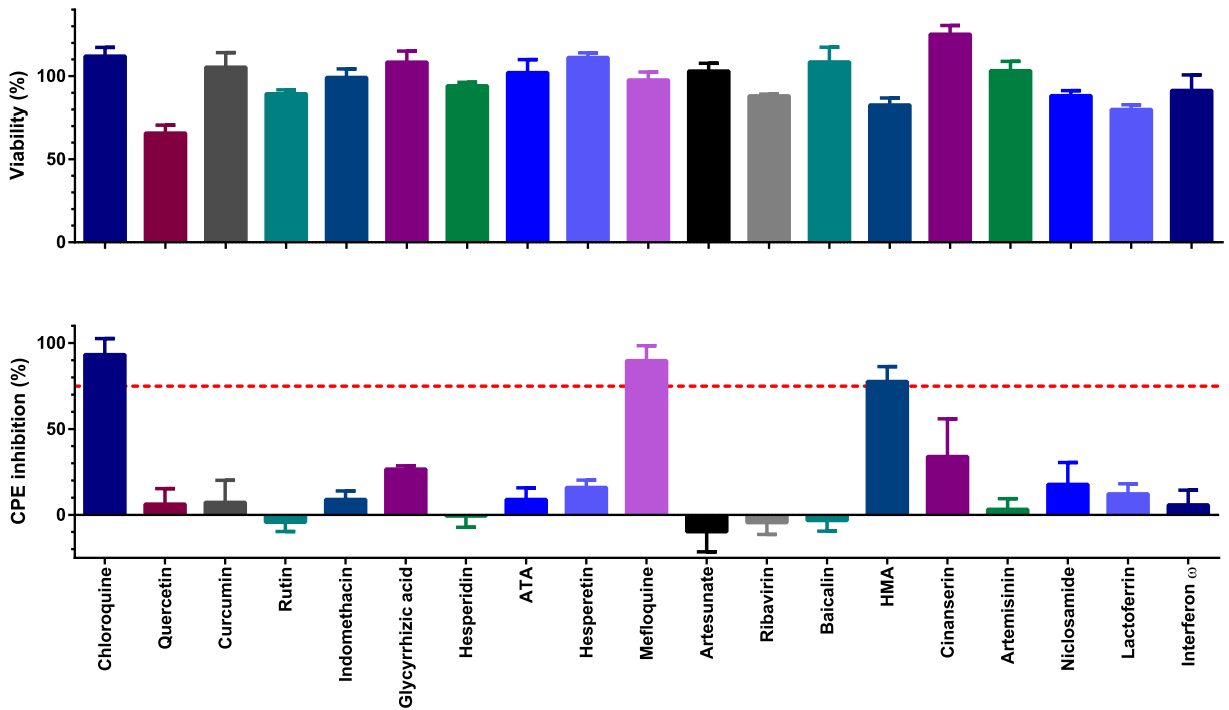


Fig. 1. Resazurin-based cytotoxicity and feline coronavirus CPE inhibition screening of selected compounds. Each treatment was performed in triplicate and repeated in three independent experiments. Results represent Mean  $\pm$  SE. ATA, aurointricarboxylic acid; HMA, hexamethylene amiloride. Dotted line = 75% inhibition.

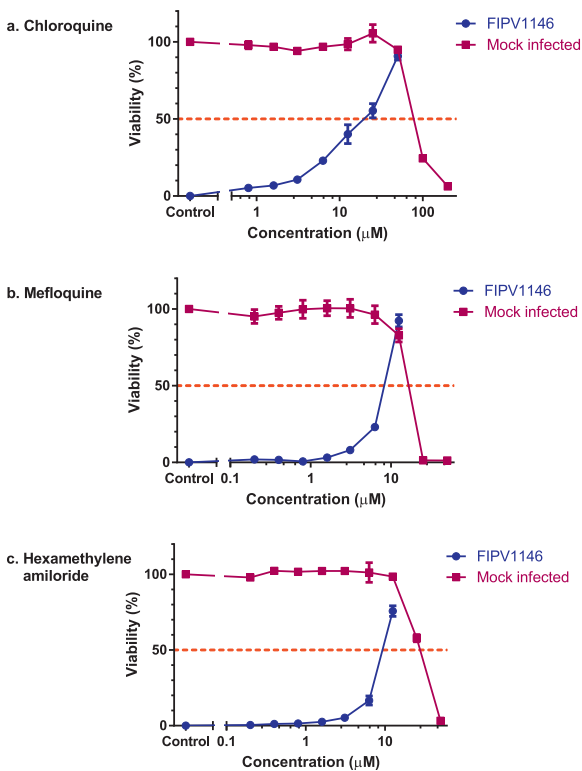


Fig. 2. Results of antiviral titration for (a) chloroquine, (b) mefloquine, and (c) hexamethylene amiloride using resazurin-based CPE inhibition assay. Results represent Mean  $\pm$  SE.

a concentration-dependent decrease in plaque number, with high concentrations completely inhibiting macroscopic plaque formation. For all compounds plaque morphology was similar between treated and untreated wells however plaque size was smaller in treated versus untreated wells.

During the virus yield reduction assay cells were monitored for the development of CPE using phase contrast microscopy. It was noted that infected and uninfected cells treated with chloroquine, mefloquine, or hexamethylene amiloride displayed characteristic morphological changes. These changes consisted of a large number of variably sized cytoplasmic (predominantly perinuclear) inclusions in addition to the presence, in some cells, of an increased number of cytoplasmic vacuoles. To investigate the nature of these inclusions, separate wells were stained with 33  $\mu$ g ml<sup>-1</sup> neutral red in DMEM for 2 h. These inclusions appeared to accumulate the vital dye neutral red following suggesting they were likely dilated endosomes/lysosomes (Fig. 4).

### 3.2. Preliminary studies on the antiviral mechanism of action of identified compounds

Using a virucidal suspension assay no virucidal effects were seen for chloroquine, mefloquine, or hexamethylene amiloride, with the infectivity of virus suspensions exposed to the compounds not significantly different from virus incubated with media alone.

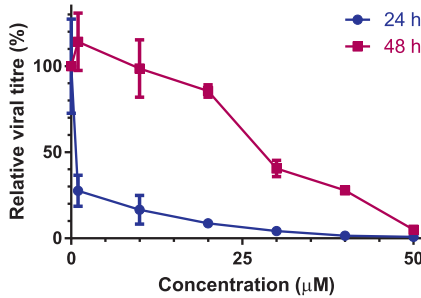
The effect of time of addition on the antiviral activity of selected compounds was assessed using a modification of

**Table 2**

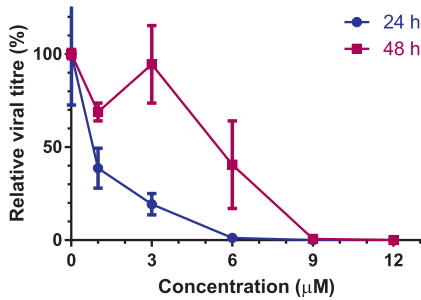
IC50, CC50, and SI values for chloroquine, mefloquine, and hexamethylene amiloride as determined using the resazurin-based CPE inhibition assay. IC50 and CC50 values given with 95% confidence intervals.

Compound	IC50 (μM)	CC50 (μM)	SI
Chloroquine	16.63 (14.44–19.15)	82.31 (76.66–88.38)	4.95
Mefloquine	7.89 (7.50–8.31)	15.13 (13.69–18.05)	1.92
Hexamethylene amiloride	9.38 (8.99–9.79)	26.50 (25.42–27.63)	2.82

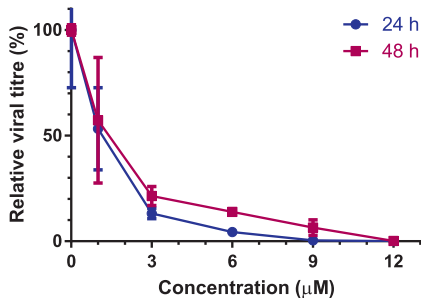
**a. Chloroquine**



**b. Mefloquine**



**c. Hexamethylene amiloride**



**Fig. 3.** Results of virus yield reduction assay for chloroquine, mefloquine, and hexamethylene amiloride. Titre of untreated control is defined as 100%. Each treatment was performed in triplicate and repeated in two independent experiments. Data represent Mean ± SE.

**Table 3**

Calculated IC50 and SI values for chloroquine, mefloquine, and hexamethylene amiloride using the virus yield reduction assay. IC50 values given with 95% confidence intervals.

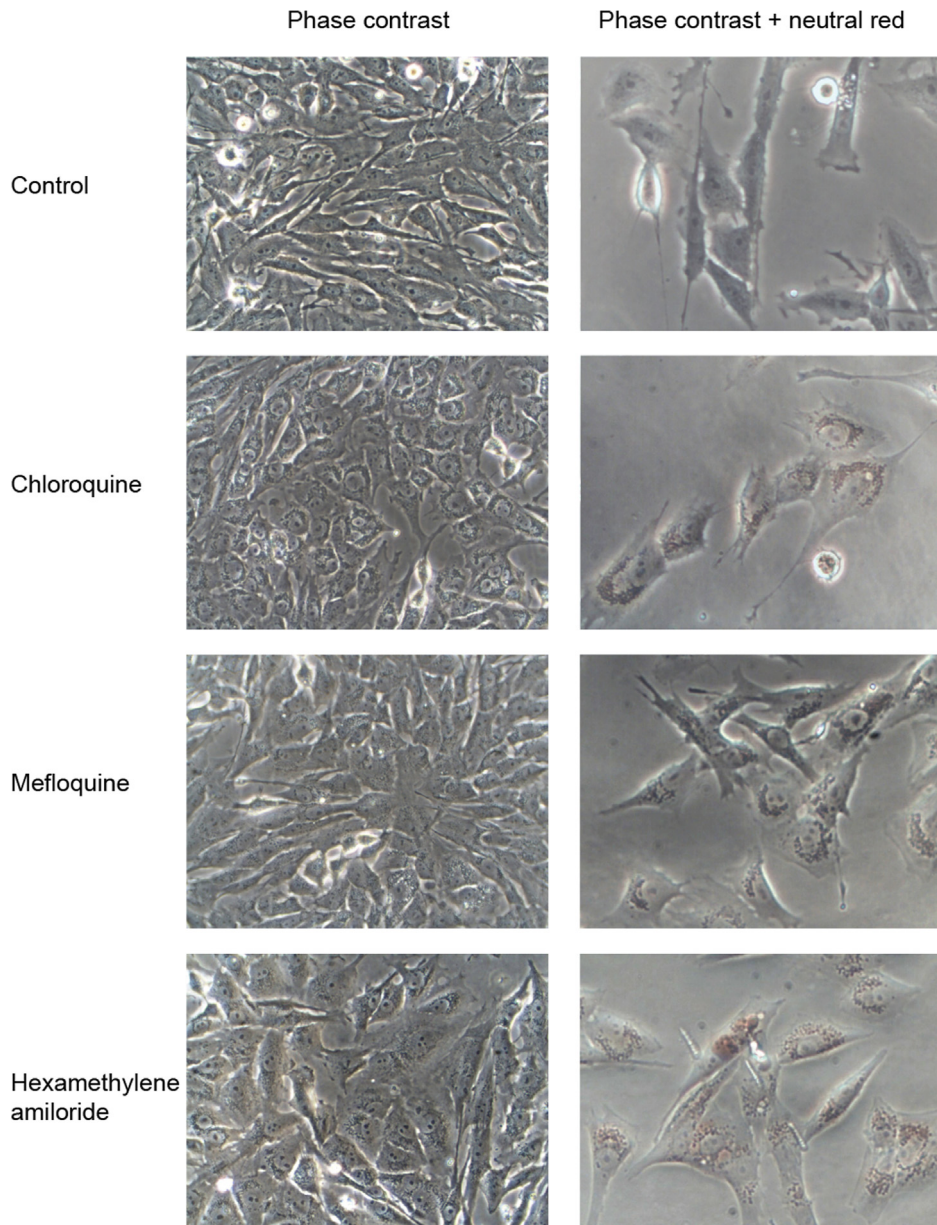
Compound	24 hpi		48 hpi	
	IC50 (μM)	SI	IC50 (μM)	SI
Chloroquine	0.38 (0.096–1.50)	217.60	28.87 (25.17–33.12)	2.85
Mefloquine	0.74 (0.32–1.73)	20.45	5.71 (4.43–7.36)	2.65
Hexamethylene amiloride	1.07 (0.66–1.73)	24.77	1.23 (0.71–2.14)	21.54

the resazurin-based CPE inhibition assay and through IFA of viral protein expression. Based on the CPE inhibition assay maximum antiviral effect was seen when compounds were added prior to or concurrent with infection, following which there was a time-dependent reduction in CPE inhibition (Fig. 5). For all tested compounds CPE inhibition remained greater than 50% when compounds were added at the latest tested time point of 6 h post-infection.

The CPE inhibition assay encompasses multiple rounds of viral replication. To further elucidate the stage of viral replication affected by test compounds a single replication cycle IFA-based assay was conducted which confirmed that, based on viral antigen expression, all three compounds possess antiviral properties when added prior to, or at the time of infection. Furthermore all compounds displayed a time of addition dependent reduction in antiviral effect; however the extent and timing of this reduction varied. The inhibitory effect of chloroquine was reduced, based on an increase in the percentage of FCoV antigen positive cells, when added at any time post-infection (Fig. 6). A similar result was seen for hexamethylene amiloride, although in this case a significant increase in the number of infected cells was not seen until compound addition was delayed until 1 hpi. In contrast, mefloquine remained effective when added up to 5 hpi suggesting it may act at a later stage of viral replication than chloroquine and hexamethylene amiloride.

**3.3. Efficacy of identified compounds against different FCoV biotypes**

The efficacy of the three identified candidate compounds was tested against FCoV FECV1683, a serotype II enteric biotype FCoV. Comparison of the virus control (no treatment) wells showed FCoV FIPV1146 infection resulted in more pronounced CPE over the 72 h infection period compared to FCoV FECV1683. Pre-treatment with chloroquine, mefloquine, or hexamethylene amiloride provided a degree of protection against strain FCoV FECV1683. Pre-treatment with hexamethylene amiloride provided protection against virus induced CPE that was similar for the two strains, with a reduction in CPE of 89.5% and 86.0% for FCoV FIPV1146 and FECV1683 respectively. Both chloroquine and mefloquine however were more effective against FCoV FIPV1146 than FECV1683, with CPE inhibition for chloroquine of 76.9% for versus 63.8%, and for mefloquine 79.0% versus 67.5% for strains FIPV1146 and FECV1683 respectively.



**Fig. 4.** Representative micrographs of morphological changes induced by treatment with chloroquine, mefloquine, and hexamethylene amiloride. Treated cells showed increased numbers of variably sized cytoplasmic (predominantly perinuclear) inclusions which accumulated the vital dye neutral red.

#### 4. Discussion

This study identifies three compounds (chloroquine, mefloquine, and hexamethylene amiloride) demonstrating a marked inhibitory effect on FCoV replication *in vitro* by significant reductions in virus induced CPE and viral titres at low micromolar concentrations when present during the early stages of viral replication. An antiviral effect of chloroquine had previously been demonstrated against FCoV, and hexamethylene amiloride had previously demonstrated efficacy against other coronaviruses, however this

is the first demonstration of antiviral efficacy of mefloquine against a coronavirus.

Initial compound screening was performed using a CPE inhibition assay, with subsequent virus yield reduction assays and plaque reduction assays used for confirmatory testing. For the effective compounds the IC<sub>50</sub> values, and corresponding selectivity index, varied with the assay method utilised. This is not unexpected given the assays measure different endpoints, and has been reported for other antiviral drugs such as the retroviral protease inhibitor saquinavir where the reported IC<sub>50</sub> calculated



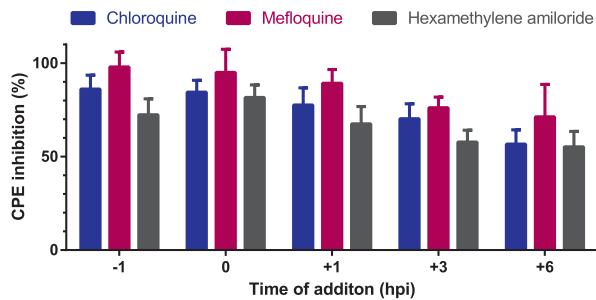


Fig. 5. Effect of time of addition on CPE inhibition for chloroquine, mefloquine, and hexamethylene amiloride. Each treatment was performed in triplicate and repeated in three independent experiments. Data represent Mean  $\pm$  SE.

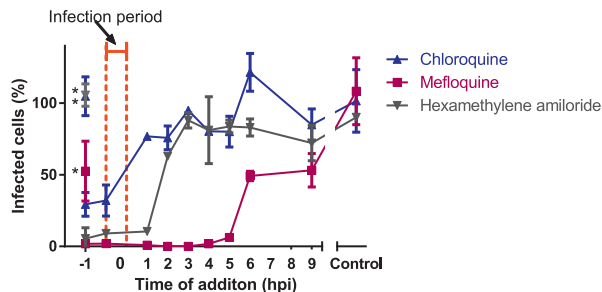


Fig. 6. Effect of time of compound addition on viral antigen expression for chloroquine, mefloquine, and hexamethylene amiloride. Results represent Mean of duplicate wells  $\pm$  SD. For ease of visualisation, samples treated with compounds only for the pre-infection period are not shown by connecting lines and are marked with an asterisks (\*). For all other treatments the added compounds remained for the duration of the experiment.

based on production of viral p24 antigen is approximately 30-fold lower than that based on production of mature virions (Buss and Cammack, 2001). Similarly variation in assay conditions may result in the calculation of significantly different IC<sub>50</sub> values. The concentration–response curve of chloroquine against SARS-CoV determined using a PCR based virus yield reduction assay was shown to shift considerably to the right when viral genome copies were assayed 3 days post-infection compared to 1 day post-infection (Keyaerts et al., 2004). A similar finding was noted in the current study for both chloroquine and mefloquine, with differences in potency reported with the TCID<sub>50</sub> based virus yield reduction assay performed at 24 and 48 hpi, however this was not seen for hexamethylene amiloride.

Two compounds, ribavirin and rFeIFN- $\omega$ , which had previously demonstrated *in vitro* efficacy against FCoV, failed to demonstrate significant inhibition of CPE during screening. For both compounds these discordant results are likely attributable to testing at concentrations below their useful therapeutic range and variations in assay conditions and sensitivity compared with previous work. The screening concentration of compounds used in this study was determined based on cytotoxicity testing to achieve cell viability greater than 80%. Previous studies with ribavirin demonstrated IC<sub>50</sub> values of 41.7  $\mu\text{g ml}^{-1}$

(170  $\mu\text{M}$ ) (Barlough and Scott, 1990) based on a visual assessment of protection from cytopathic effect and 2.5  $\mu\text{g ml}^{-1}$  (10.2  $\mu\text{M}$ ), based on the reduction of extracellular viral titre (Weiss and Oostrom-Ram, 1989). The concentration used for screening (2.5  $\mu\text{M}$ ) was therefore more than 60 times lower than the IC<sub>50</sub> previously calculated based on a similar assay endpoint. From the results of the current study, virus yield reduction assays appear to provide a more sensitive assessment of antiviral efficacy than CPE inhibition assays, with the IC<sub>50</sub> values calculated based on viral titre reduction significantly lower than those calculated based on CPE inhibition for all compounds. A small antiviral effect of ribavirin cannot therefore be ruled out based on the current findings, as although the tested concentrations did not provide protection against virus induced CPE, it may have been associated with a reduction in extracellular viral titre. The practical relevance of such a small antiviral effect is questionable, particularly given the known toxicity profile of this compound in cats.

For rFeIFN- $\omega$  reductions in viral titres of 0.2–1.2 logs have been reported when CRFK cells were treated with 50,000 U ml<sup>-1</sup> 1 h post-infection (Truyen et al., 2002) and 0.5–0.6 logs when FCWF cells were pre-treated with 100–100,000 U rFeIFN- $\omega$  (Mochizuki et al., 1994). Protection from CPE was not seen in the current study when cells were pre-treated with rFeIFN- $\omega$  at 100 U ml<sup>-1</sup>, a concentration significantly lower than that previously shown to be effective using the same virus strain and cell line (Truyen et al., 2002). The tested concentration was however similar to that used by Mochizuki et al. (1994). This apparent lack of efficacy in this case may reflect differences in the drug exposure and infection conditions, the viral isolate tested, or an intrinsic enhanced susceptibility to the antiviral effects of interferon in FCWF cells compared to CRFK cells as used in this study (Weiss and Toivio-Kinnucan, 1988). Alternatively it may be that, as suggested for ribavirin, virus yield reduction assays provide a more sensitive assessment of antiviral effects than CPE inhibition assays, and that the screening method utilised failed to identify mild antiviral effects.

A number of different mechanisms of action have been suggested to account for the antiviral properties of the compounds identified in this study against other viruses. For chloroquine antiviral effects have been ascribed to inhibition of glycosylation of viral proteins (Savarino et al., 2004) or cellular receptors for viral attachment (Vincent et al., 2005), inhibition of glycoprotein expression (Dille and Johnson, 1982), or inhibition of endosome mediated viral entry (Savarino et al., 2003). The antiviral effect of mefloquine against JC virus has been postulated to be due to its action as an adenosine mimetic (Brickelmaier et al., 2009), while for hexamethylene amiloride it has been suggested antiviral properties against different viruses may arise through competitive inhibition of viral RNA polymerase (Gazina et al., 2011), an indirect mutagenic effect (Levi et al., 2010), or inhibition of viroporins (Wilson et al., 2006).

Interestingly all three compounds showing marked antiviral efficacy against FCoV in this study resulted in similar morphological changes in cells exposed to

sub-toxic concentrations. Increased numbers of variably size cytoplasmic inclusions that accumulate the viral dye neutral red suggests these compounds result in a perturbation of the normal endocytic pathway in CRFK cells. Alterations in the endocytic pathway have previously been reported for chloroquine (Dean et al., 1984), mefloquine (Labro and Babin-Chevaye, 1988), and for amiloride and some of its derivatives (Dutta and Donaldson, 2012). This suggests a common physiological effect on treated cells for all three candidate antivirals and possibly a shared mechanism of action. Viruses are known to usurp a variety of host endocytic pathways for cell entry and intracellular movement and inhibition of these pathways may be a useful therapeutic approach. Although targeting a cellular pathway may be associated with an increased risk of toxicity, if that pathway is critical for viral replication this approach may slow or limit the development of resistance.

Time of addition studies demonstrated all compounds were most effective when added prior to infection, suggesting a mechanism of action involving early stages of viral replication. The CPE inhibition based time of addition assay involved infection at low MOI with a 72 h infection period, allowing for multiple rounds of viral replication. As a result of this, even with the delayed addition of compounds, cells uninfected by the original inoculum are effectively pre-treated prior to challenge with progeny virions produced during the primary replication cycle. Using an IFA-based time of addition study involving a single replication cycle we were able to further clarify the effect of time of addition, and refine the possible stage of the viral life cycle targeted by each compound. Based on the IFA results chloroquine was effective only if present at the time of infection, supporting the hypothesis that chloroquine acts during cell entry for FCoV FIPV1146, possibly through inhibition of endosomal pH (Takano et al., 2008). Hexamethylene amiloride and mefloquine provided significant antiviral effects when compound addition was delayed for up to 1 and 5 hpi respectively, suggesting that if the antiviral effects of these compounds arise through perturbation of endosomal function, the effects occur at different stages of the viral life cycle. Alternatively distinct mechanisms of action may account for the observed effects of these compounds, as suggested for other viruses.

There is limited published pharmacokinetic or safety data to inform the potential therapeutic application of the identified compounds in cats and given the relatively low SI of all three compounds consideration must be given to their *in vivo* safety in this species. The human approved pharmaceuticals chloroquine and mefloquine are generally considered well-tolerated drugs, albeit with a narrow therapeutic index, while the clinical use of hexamethylene amiloride has not been reported. Pharmacokinetic data available for chloroquine and mefloquine in humans would suggest that effective plasma concentrations could be achieved at standard therapeutic doses (Pussard and Verdier, 1994; Simpson et al., 1999). Chloroquine has been shown to accumulate in leukocytes, where the concentration may be two orders of magnitude greater than that of plasma (Mackenzie, 1983), with the highest concentration

reported in monocytes (French et al., 1987). Thus therapeutic concentrations may be attained in the target cells of virulent biotype FCoVs at relatively low plasma concentrations, minimising the risk of dose-dependent adverse effects. Mefloquine is known to accumulate within brain parenchyma at concentrations approximately 10–30 times higher than found in serum, with tissue concentrations of up to 50  $\mu\text{M}$  reported (Nevin, 2009; Pham et al., 1999). Mefloquine may therefore be useful in the treatment of dry (non-effusive) FIP, where CNS lesions are common (Pedersen, 2009) although the potential for neurotoxicity must be considered. Although the concentration of mefloquine achieved in the CNS is greater than the CC50 of this compound in immortalised feline kidney cells, in humans this tissue concentration is achievable at therapeutic doses, despite *in vitro* data in human cells showing a CC50 approximately equal to that determined in the current study (Brickelmaier et al., 2009). It may be therefore that the more static cell population of the CNS is more refractory to the toxic effects of mefloquine than mitotically active immortalised cells.

## 5. Conclusion

This study has identified three compounds demonstrating marked *in vitro* inhibition of FCoV in an immortalised cell line at low micromolar concentrations, including the first demonstration of antiviral effects of mefloquine against a coronavirus. Although the low SI of the three compounds may limit their therapeutic utility, these preliminary studies open the way for further investigation and potential optimisation of these compounds as antiviral agents.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2014.10.030>.

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