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Identification and characterisation of small molecule inhibitors of feline coronavirus replication

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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.

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.

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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_2 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*	25 μM
Quercetin*	10 μM
Curcumin†	10 μM
Rutin trihydrate†	25 μM
Indomethacin†	10 μM
Glycyrrhizic acid*	25 μM
Hesperidin†	50 μM
Aurintricarboxylic acid*	2.5 μM
Hesperetin†	50 μM
Mefloquine hydrochloride*	10 μM
Artesunate*	1 μM
Ribavirin*	2.5 μM
Baicalin hydrate†	10 μM
Hexamethylene amiloride†	10 μM
Cinanserin†	20 μM
Artemisinin*	25 μM
Niclosamide†	0.25 μM
Lactoferrin*	0.5 mg ml^{-1}
Recombinant feline interferon ω‡	100 units ml^{-1}