A novel corneal explant model system to evaluate antiviral drugs against feline herpesvirus type 1 (FHV-1)

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Feline herpesvirus type-1 (FHV-1) is the most common viral cause of ocular surface disease in cats. Many antiviral drugs are used to treat FHV-1, but require frequent topical application and most lack well-controlled in vivo studies to justify their clinical use. Therefore, better validation of current and novel treatment options are urgently needed. Here, we report on the development of a feline whole corneal explant model that supports FHV-1 replication and thus can be used as a novel model system to evaluate the efficacy of antiviral drugs. The anti-herpes nucleoside analogues cidofovir and acyclovir, which are used clinically to treat ocular herpesvirus infection in cats and have previously been evaluated in traditional two-dimensional feline cell cultures in vitro, were evaluated in this explant model. Both drugs suppressed FHV-1 replication when given every 12 h, with cidofovir showing greater efficacy. In addition, the potential efficacy of the retroviral integrase inhibitor raltegravir against FHV-1 was evaluated in cell culture as well as in the explant model. Raltegravir was not toxic to feline cells or corneas, and most significantly, inhibited FHV-1 replication at 500 µM in both systems. Importantly, this drug was effective when given only once every 24 h. Taken together, our data indicate that the feline whole corneal explant model is a useful tool for the evaluation of antiviral drugs and, furthermore, that raltegravir appears a promising novel antiviral drug to treat ocular herpesvirus infection in cats.

INTRODUCTION

Alphaherpesvirinae, a subfamily of Herpesviridae, cause disease in many species including humans and animals of veterinary importance. These large DNA viruses are characterized by (i) a short replication cycle, (ii) infection through mucosal and epithelial surfaces, and (iii) induction of lifelong latency, primarily in neurons (Davison et al., 2009). Feline herpesvirus type 1 (FHV-1), a varicello-virus of this subfamily, has an estimated 90–97 % seroprevalence in cats (Maggs et al., 1999). Furthermore, about 80 % of FHV-1-infected cats remain latently infected for life and 45 % of these animals intermittently shed virus (Gaskell & Povey, 1977). Notably, FHV-1 is the most common viral cause of ocular surface disease in cats, primarily inducing acute corneal ulceration and the development of chronic stromal keratitis (Gaskell et al., 2007; Gould, 2011; Stiles, 2014).

While several FHV-1 vaccines have widespread use and can reduce clinical signs, they only confer partial protection against infection, viral shedding, and latency load (Gaskell et al., 2007). Therefore many cats still develop FHV-1-associated ocular disease despite vaccination. Treatment requires a multifaceted approach tailored both to the individual patient and to the owner, although all current treatment regimens rely on the use of antiviral drugs. Despite the fact that many antiviral drugs, primarily nucleoside analogues, have been tested in vitro for efficacy against FHV-1, no antiviral agent has ever been designed specifically for either FHV-1 or for use in cats (Galle, 2004; Maggs, 2010; Stiles, 2014). Moreover, many antivirals require frequent topical application and most lack well-controlled in vivo studies to justify their clinical use (Gould, 2011). Cidofovir is one of the few nucleosides analogues that have been tested in cats under well-controlled experimental conditions and showed a 33 % reduction in FHV-1 viral titres in FHV-1-infected, cidofovir-treated cats compared to FHV-1-infected, untreated control cats (Fontenelle et al., 2008). Another nucleoside analogue frequently used to inhibit alphaherpesvirus replication is acyclovir. Yet, there are contrasting indications for the use of acyclovir to treat ocular...
FHV-1 infections and the reported effective concentration 50 % (EC$_{50}$) of 57.9 μM (Maggs & Clarke, 2004) is approximately 50–500 × greater than the range of acyclovir of 0.07–0.97 μM against 77 different herpes simplex virus type 1 (HSV-1) isolates (Sangdara & Bhattarakosol, 2008). Additionally, its oral produg, valacyclovir, is toxic to cats (Nasisse et al., 1997). Thus acyclovir is not generally recommended for use in cats, although topical acyclovir may be useful to treat drug-resistant ulcers (Rand, 2006), and one study reports the successful topical administration of acyclovir, five times a day for a period of 21 days, to cats with ocular FHV-1 (Williams et al., 2005).

Recently, Yan et al. (2014) reported that small molecule human immunodeficiency virus (HIV) integrase inhibitors were capable of blocking replication of all three families of herpesviruses by targeting the polymerase-helicase complex, resulting in inhibition of viral DNA replication. Furthermore, it was also recently found that raltegravir, a currently available retroviral integrase inhibitor approved by the US Food and Drug Administration (FDA) in 2007 (Summa et al., 2008), was able to interfere with UL42, the DNA polymerase accessory factor of the human alphaherpesvirus HSV-1 (Zhou et al., 2014). Based on the fact that (i) the FHV-1-encoded UL42 shares 24 % amino acid sequence similarity with HSV-1 UL42 (Zhukovskaya et al., 2014) and (ii) raltegravir appears safe to administer systemically to cats (Boesch et al., 2015), raltegravir could be an attractive novel antiviral drug to treat ocular herpesvirus infection in cats.

The lack of an effective and standardized antiviral therapy to treat feline ocular herpes infections largely stems from the limitation of the traditional two-dimensional in vitro cell culture system and the logistical complications of working with live cats, such as the high cost of acquiring these animals from approved vendors, the high cost of care, and ethical considerations. Organ explant model systems are valuable tools as they preserve the tissue cytoarchitecture to more accurately reproduce what occurs at the organizational level (Resau et al., 1991; Grivel & Margolis, 2009) and, therefore, are a useful steppingstone to bridge in vitro and in vivo evaluation of drugs, including antiviral drugs. Indeed, the valuable use of explants in drug discovery has been reported for a variety of organs, including intestines (Leushacke & Barker, 2014), skin (Mathes et al., 2014), and the central nervous system (Daviaud et al., 2013). Furthermore, the use of discarded material is less expensive than live animal use and reduces the number of experimental animals needed, as outlined by the 3R concept (Russell & Burch, 1959).

Therefore, the aim of the present study was to use a feline whole corneal explant model system to evaluate the efficacy of commonly used and novel promising antiviral drugs against FHV-1 infection. Our salient findings were that the anti-herpes nucleoside analogues cidofovir and acyclovir, as well as the retroviral integrase inhibitor raltegravir, could significantly inhibit FHV-1 replication. These data provide additional support to the topical use of cidofovir and acyclovir, and suggest that raltegravir could prove a novel effective antiviral to treat FHV-1-induced ocular herpesvirus infection.

**RESULTS**

The whole corneal explant model supports FHV-1 infection

The whole corneal explant model system was established as shown in Fig. 1(a). In order to optimize the viral growth conditions, corneas were infected with FHV-1 and cultured at either 34 °C or 37 °C. At 24 h post-infection (hpi), viral growth was approximately fourfold higher at 34 °C compared to 37 °C, as assessed by qPCR on corneal tissue for viral genome replication and plaque assay of supernatants for extracellular viral titers (Fig. 1b). Consequently, all further experiments were done with corneas cultured at 34 °C. In addition, hematoxilin-eosin (H&E) stainings of corneal cross-sections showed that FHV-1-infected corneas exhibited epithelial damage, characterized by epithelial thinning and detachment, while mock-infected corneas displayed a normal, multi-layer stratified squamous epithelium (Fig. 1c). Immunofluorescence (IF) of corneal cross-sections using an anti-FHV-1 monoclonal antibody demonstrated the presence of viral antigen primarily in the central region of the epithelium of FHV-1-infected corneas (Fig. 1c), similar to the distribution of FHV-1 in feline corneas in vivo (Nasisse et al., 1989). In contrast, and as expected, no viral antigen staining was found in mock-infected corneas (Fig. 1c). Taken together, these results show that the whole corneal explant model system supports FHV-1 replication and that this replication is more efficient when corneas are cultured at 34 °C.

The anti-herpes nucleoside analogues cidofovir and acyclovir inhibit FHV-1 replication in the whole corneal explant model

To start evaluating the efficacy of antiviral drugs against FHV-1 in this new explant model system, cidofovir and acyclovir were chosen since these two anti-herpes nucleoside analogues are clinically used to treat ocular herpesvirus infection in cats and have previously been evaluated in traditional two-dimensional feline cell cultures (Maggs & Clarke, 2004). The EC$_{50}$ of these two antivirals was first determined in FHV-1-infected Crandell Reese Feline Kidney (CFRK) cells to decide on the concentration to be used in the corneal explant model system. EC$_{50}$ values of 7.1±2.1 μM for cidofovir and 78.6±6.2 μM for acyclovir were found (Fig. 2a), similar to what has been reported previously (Maggs & Clarke, 2004). Sets of matched corneas were then infected with 3×10$^3$ plaque forming units (PFU) FHV-1 for 2 h, after which one cornea was incubated with the antiviral drug at approximately twice the EC$_{50}$ (i.e. 16.2 μM for cidofovir and 181 μM for acyclovir), while the other cornea was left untreated. Medium with the same concentration of antiviral drug was refreshed every 12 h,
broadly based on the in vivo half-life of these drugs which is ~2–3 h in adult humans (Cundy et al., 1995; Kimberlin & Whitley, 2007). Medium without antiviral drugs was used to refresh control corneas every 12 h. Corneas were collected 48 h later for further analyses.

Upon treatment with cidofovir and acyclovir, FHV-1-infected corneas showed a significant reduction in viral load of 98±3 % and 73±20 %, respectively, when compared to untreated FHV-1-infected corneas (Fig. 2b). Evaluating the extracellular virus production in the supernatants, which were collected every 12 h when media was refreshed, showed that (i) treatment with either antiviral resulted in a significantly reduced virus production starting at 36 h post-treatment and (ii) even resulted in the complete absence of extracellular infectious virus at 48 h post-treatment with cidofovir (Fig. 2c). IF staining of cross-sections from FHV-1-infected, antiviral-treated corneas generally showed an overall reduced viral antigen-positive staining in the epithelium when compared to untreated FHV-1-infected corneas (Fig. 2d). When analysing the thickness of the epithelium of H&E-stained corneal cross-sections, the following observations were made. FHV-1-infected, untreated control corneas were observed to have significantly thinner epithelial layers than those of the mock-infected, non-matched corneas with the cidofovir, but not the acyclovir experiments (Fig. 2e). This inconsistent thinning of the epithelial layer between FHV-1-infected corneas, which is most likely attributed to inter-animal variability in corneal thickness, highlights the importance of analysing drug efficacy data within the matched control. In FHV-1-infected, antiviral-treated corneas, (i) the epithelial layers of antiviral-treated corneas were significantly thicker than those of the matched FHV-1-infected, untreated control corneas and (ii) no significant difference in epithelial thickness was seen compared to the mock-infected, non-matched corneas (Fig. 2e).

Taken together, these results show that both the antiviruses nucleoside analogues cidofovir and acyclovir can inhibit FHV-1 replication in the whole corneal explant model, with cidofovir having a more pronounced effect compared to acyclovir, and that antiviral drug treatment...

Fig. 1. The feline whole corneal explant model is susceptible for feline herpesvirus type 1 infection. (a) Whole eyes were obtained from cats euthanized for reasons unrelated to this study (i). Corneas were removed from the eye after discarding the lens and iris (ii). The cornea was placed epithelial side down and the inner cavity was filled with 1 % low-melting-point agarose in DMEM (iii). Corneas were placed epithelial side up in 12-well plates and covered with media (iv). (b) Paired corneas (n=1) from the same cat were infected with 3×10⁵ PFU FHV-1 and cultured at either 34 °C or 37 °C for 24 h. Viral load in the cornea at each temperature was assessed by qPCR and normalized to the cornea cultured at 34 °C by the ddCt method and set to 100 %, and produced infectious virus in the supernatants was assessed by standard plaque assays. (c) Mock- or FHV-1-infected corneal cross-sections were stained with hematoxilin and eosin (H&E), or with monoclonal anti-FHV-1 antibodies followed by Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained with DAPI. Bars, 100 µm.
appears to limit the corneal damage (i.e. reduction of epithelial thickness) observed after FHV-1 infection.

**The retroviral integrase inhibitor raltegravir inhibits FHV-1 replication in both CRFK cells and the whole corneal explant model**

Based on the recent reports that retroviral integrase inhibitors are also effective against herpesviruses (Yan et al., 2014; Zhou et al., 2014), we decided to explore the potential of this class of antiviral drugs against FHV-1 in the whole corneal explant model. The retroviral integrase inhibitor raltegravir was used as it is currently approved for use in humans for the treatment of human immunodeficiency virus infection (Summa et al., 2008), and was recently shown to be safe for use in cats (Boesch et al., 2015). Since this drug has never been evaluated for FHV-1, it was initially tested for its effect on FHV-1 replication in the traditional two-dimensional cell culture system using CRFK cells. First, cytotoxicity of the drug was assessed by incubating CRFKs with increasing concentrations of raltegravir for 24 h. No effect on viability was observed up to 500 µM (Fig. 3a). At higher concentrations, i.e. 1000 and 2000 µM raltegravir, a significant reduction in cell viability of around 15 % was found (Fig. 3a). No effect on viability was seen when CRFKs were incubated with volume-matched amounts of DMSO (Fig. 3a).

Next, antiviral activity of raltegravir was assessed by determining both plaque numbers and sizes in FHV-1-infected CRFKs treated with this antiviral drug at various concentrations ranging from 0 µM (control) up to 500 µM. A dose-dependent decrease in relative plaque numbers, indicative of viral infectivity, was observed, which reached significance at 500 µM (Fig. 3b), and likewise, a dose-dependent decrease in relative plaque sizes, indicative of cell-to-cell spread was observed, which reached significance at 200 µM (Fig. 3b). To further corroborate these findings, CRFKs were infected with FHV-1 for 2 h at 37 °C, washed, and treated with 500 µM raltegravir for 24 h. Viral genome copies in FHV-1-infected CRFKs were determined using qPCR and release of infectious virus particles were determined using standard viral plaque assays. A significant decrease in both viral genome copies and extracellular infectious virus was found in raltegravir-treated compared to untreated, FHV-1-infected CRFK cells after 24 h (Fig. 3c). Finally, a time kinetic experiment was also performed to explore the efficacy of 500 µM raltegravir on FHV-1 replication when administered at different time points (0–48 hpi). A significant reduction in both viral genome copies and extracellular infectious virus was found in treated versus untreated, FHV-1-infected CRFKs at all time points tested (Fig. 3d). Taken together, these data show that in the traditional two-dimensional cell culture system, raltegravir is effective at inhibiting FHV-1 replication (i) in a dose-dependent manner, with significance at 500 µM, and (ii) even when applied 48 h after FHV-1 infection.

Because of these promising results observed in CRFK cultures, we next wanted to explore the effects of raltegravir against FHV-1 in the physiologically relevant, whole corneal explant model system. In line with our studies on the efficacy of the anti-herpes nucleoside analogues, the EC\textsubscript{50} of raltegravir was first determined in order to decide on the concentration to be used in the corneal explant model system. The EC\textsubscript{50} of raltegravir was found to be 215.6 ± 7.7 µM (Fig. 4a) and consequently, 500 µM was used for experiments in the corneal explant model system since this (i) equals approximately twice the EC\textsubscript{50}, and (ii) corresponds with the significant reduction in FHV-1 replication as observed in the CRFK cultures. Next, the cytotoxicity of this concentration of raltegravir was evaluated using matched corneas, and no visual histological changes in the epithelial layer, nor any difference in epithelial thickness were observed in H&E-stained cross-sections of raltegravir-treated versus untreated corneas (Fig. 4b). Additionally, no TUNEL-positive epithelial cells, indicative of cell death, were detected following raltegravir treatment (Fig. 4c). This absence of staining was not due to a technical error as TUNEL-positive cells were readily visible in a control feline cornea that was treated with the enzyme DNase I (Fig. 4c).

After confirming that raltegravir was not toxic for the corneal epithelium, its antiviral properties were further studied in the corneal explant model system. To this end, matched FHV-1-infected corneas were treated with 500 µM raltegravir or left untreated (control) every 24 h for 2 days. The interval of treatment of 24 h was broadly based on the in vivo half-life of this drug, which is reported to be 7–12 h in humans (Hicks & Gulick, 2009). A significant reduction in both viral genome copies and extracellular infectious virus was found (Fig. 5a), and a generally reduced viral antigen-positive staining in corneal cross-sections (Fig. 5b) was observed in raltegravir-treated versus untreated FHV-1-infected corneas. Moreover, in the H&E-stained corneal cross-sections, the epithelial layers of the corneas following raltegravir treatment were observed to be significantly thicker compared to those of the matched FHV-1-infected, untreated controls (Fig. 5c). When comparing the results of these FHV-1-infected, untreated corneas, with the non-matched, mock-infected control corneas shown in Fig. 2(e), a significant reduction in the average thickness of the epithelial layer in FHV-infected corneas was observed (Student’s t-test, p=0.03). In contrast, no significant difference was found between the FHV-1-infected, raltegravir-treated corneas and the non-matched, mock-infected control corneas shown in Fig. 2(e) (Student’s t-test, p=0.07). Taken together, these results demonstrate that in the whole corneal explant model system raltegravir significantly inhibits FHV-1 replication and prevents FHV-1-induced epithelial thinning.
DISCUSSION

FHV-1 is the most common viral cause of ocular disease in cats, and while several antiviral drugs are used to treat ocular FHV-1, many require frequent topical application and most lack well-controlled in vivo studies to justify their clinical use. In the present study, we reported on the development of a feline whole corneal explant model that supports FHV-1 replication and that was used to evaluate the efficacy of antivirals against FHV-1 in a physiologically relevant model.

In general, the development and testing of novel drugs is a complex and expensive process. From 1991–2000, only an 11% success rate in bringing new drugs to the market was reported, with substantial failure occurring during Phase I–III clinical testing. Low efficacy and toxicity are indicated as primary drivers of drug attrition (Kola & Landis, 2004) and...
**Fig. 3.** Raltegravir is not toxic to feline kidney (CRFK) cells and inhibits FHV-1 replication in this cell line (n=3). (a) Raltegravir is not toxic to CRFK cells. CRFK cells were incubated for 24 h with increasing concentrations of raltegravir or DMSO (control). Cell death was determined by flow cytometry using 7-aminoactinomycin D (7AAD) staining and is expressed relative to para-formaldehyde-fixed cells (100% cell death). (b) Raltegravir reduces plaque number and size in FHV-1-infected CRFK cells. Relative plaque number and relative plaque size of confluent CRFK cells in 24-well plates infected with 75 PFU/well FHV-1 and treated for 24 h with 0–500 µM raltegravir. (c) Raltegravir significantly reduces FHV-1 replication in CRFK cells when applied directly after infection. CRFK cells in T25 flasks were infected with 200 PFU/flask FHV-1 for 2 h, washed and subsequently incubated with ~2× the EC$_{50}$ of raltegravir (500 µM) for 24 h. Viral load in CRFK cells was assessed by qPCR and produced infectious virus in the supernatants was assessed by standard plaque assays after 24 h. (d) Raltegravir significantly reduces FHV-1 replication when applied later after infection. CRFK cells in T25 flasks were infected with 50 PFU per flask FHV-1 and treated with 500 µM raltegravir at 0, 24 and 48 hpi. Relative viral load in CRFK cells was assessed by qPCR and produced infectious virus in the supernatants was assessed by standard plaque assays following drug treatment for 24 h. Dotted line represents FHV-1-infected, untreated CRFK viral load set to 1.0. Results are presented as mean±standard deviation. *p≤0.05; **p<0.01; ***p<0.001; ****p<0.0001.
potential toxicity is seldom recapitulated in simplified in vitro models such as two-dimensional cell culture (Astashkina et al., 2012). Explant models can take advantage of ‘waste’ tissue materials left over from other groups to serve as an intermediary evaluation stage between two-dimensional cell culture and live animal work. Since explant models consist of different cell types and maintain the three-dimensional architecture, they are believed to recapitulate the in vivo-like cell heterogeneity and architecture much better than two-dimensional monocolour cultures, and as such, are better predictors of in vivo efficacy of drugs (Astashkina et al., 2012).

Recently, the development of a partial corneal explant was described to evaluate its use to study FHV-1 replication (Li et al., 2015). In these partial explants, the authors only observed virus infection at the borders where the corneal tissue was cut, but not in the centre of the cornea. This is in contrast to our findings where FHV-1 replication, as assessed by immunofluorescence staining, was primarily found in the centre of intact corneas. Several explanations can potentially account for this discrepancy. First, the study of Li et al. (2015) used the C-27 FHV-1 strain and the authors hypothesized that the inability of C-27 to properly infect corneas could be explained by viral attenuation due to prolonged passage, whereas we used another virus strain, namely FH2CS. Second, Li et al. (2015) cultured corneas at 37 °C and the temperature of culturing these explants during infection could also play a critical role. Indeed, when evaluating the effects of temperature on FHV-1 replication in feline corneal explants, we found that 34 °C supported higher FHV-1 replication than 37 °C. This can be explained by the fact that corneas are directly exposed to the outside environment and thus have a lower temperature than the general body temperature. Moreover, it has been shown that feline corneal explants have a temperature of approximately 33 °C (Roh et al., 1990), and consequently, it is reasonable to assume that ocular herpesviruses are specifically adapted to grow at these lower temperatures. Third, the use of a partial corneal explant instead of a whole explant could mask virus infection if virus replication is localized to certain regions of the corneal epithelium. In this regard, we noticed when scanning corneal cross-sections for FHV-1-positive cells that only partial regions throughout the cornea appeared positive, instead of a uniform infection of the entire corneal epithelium (data not shown). Since we infected the corneas epithelial side down, as previously described by Alekseev et al. (2012), it is possible that this method may have limited access of the virus to the small central part of the cornea that was in contact with the culture plate and could potentially explain why we did not observe a uniform infection of the epithelium. It will be interesting in future experiments to evaluate FHV-1 replication when corneas are infected epithelial side up, and whether this will result in differences compared to infection of corneas epithelial side down.

Another observation we made was that FHV-1 infection generally resulted in a thinner epithelial layer when compared to mock-infected corneas, although this was very unpredictable. A likely explanation for this inconsistency is that these comparisons were made with corneas from different cats, as the matched corneas were used to compare FHV-1 infection in the presence or absence of antiviral drugs, and consequently, emphasizes the importance of using matched corneas from the same cats in order to directly compare an experimental condition with its appropriate control condition.

To validate our novel corneal explant model as a useful tool to evaluate antiviral drugs, we first focused on the anti-herpes nucleoside analogues cidofovir and acyclovir, which are already used clinically and have been evaluated previously in cell culture (Mags & Clarke, 2004; Gould, 2011). We found that both antiviral drugs were capable of inhibiting FHV-1 replication in feline corneas, but cidofovir appeared to be more effective (higher reduction) and more consistent (smaller standard deviations) at controlling infection when compared to acyclovir. Overall, and importantly, the results in the whole corneal explant model appear to match what has been observed in vivo with these two antivirals. Indeed, cidofovir treatment at a 12 h interval and a dose of ~16 μM was quite effective at inhibiting FHV-1 replication in the corneal explant model, similarly to its antiviral effects in vivo as assessed by a reduction in viral titres in ocular swabs of experimentally infected cats (Fontenelle et al., 2008). When looking at acyclovir, much higher concentrations, ~180 μM, were needed to efficiently inhibit FHV-1 replication at a similar treatment interval in the feline corneal explant model and a much larger standard deviation was observed. Williams et al. (2005) evaluated the efficacy of topical acyclovir in 30 client-owned FHV-1-positive cats. These cats were first treated with the antibiotic chlortetracycline for 2–3 weeks and due to the lack of any improvement in ocular disease, received topical treatment with acyclovir five times per day. This resulted in a clinical recovery of ocular disease after 12 days of acyclovir treatment. However, due to the lack of control cats in that study, it is difficult to make strong conclusions regarding the efficacy of topical acyclovir in vivo. Additionally, Nasisse et al. (1997) reported that the oral prodrug of acyclovir, valacyclovir, is toxic to cats. This was likely due to the systemic toxicity of acyclovir itself. If the low bioavailability of acyclovir could be overcome, it would likely have similar toxicity. It is currently not clear if long-term topical administration of acyclovir would also be toxic to cats. Our data with the corneal explant model system suggest that acyclovir can indeed be effective to treat FHV-1-induced ocular disease, but due to the high frequency of treatment and high dosage required, as well as its potentially toxic effects, there may be more appropriate antiviral drugs for ocular herpesvirus infection in cats.

Finally, our whole corneal explant model system was used to evaluate an antiviral drug that has not previously been used to treat FHV-1 infection in general,
or FHV-1-induced ocular herpesvirus disease specifically. The antiviral drug raltegravir, is an FDA-approved retroviral integrase inhibitor which was recently shown to inhibit replication of the human alphaherpesvirus herpes simplex virus type 1 (HSV-1) (Summa et al., 2008; Zhou et al., 2014). Similar to the anti-herpes nucleoside analogues, raltegravir was also capable of inhibiting FHV-1 replication in the feline corneal explant system. It is interesting to note that this effect was observed when raltegravir was added at a 24 h interval, in contrast to the 12 h interval of the nucleoside analogues, and as such, we would like to suggest raltegravir as a novel promising antiviral drug for topical use in cats with less frequent applications. The latter is especially important since patient compliance is a common problem in the treatment of FHV-1 ocular infection and so the use of antiviral drugs that have to be given less frequently may increase the likelihood of compliance (Williams et al., 2005; Gould, 2011). Unfortunately, raltegravir was found to have a relatively high EC_{50} value which could limit its practical use against ocular FHV-1, even though we did not observe any direct toxic effects in our corneal explant system with 500 µM raltegravir, the concentration that significantly inhibited FHV-1 replication. It will be of interest to screen additional integrase inhibitors, like 1118-D-24 or XZ45, in our whole corneal explant model system to identify more potent inhibitors of FHV-1 replication with a

**Fig. 4.** Raltegravir is effective against FHV-1, but is not toxic by itself for feline corneas. (a) The EC_{50} of raltegravir (n=3). The EC_{50} value was determined to assess the potency of raltegravir to reduce FHV-1-induced cytopathic effect and was calculated based on the percent of wells showing CPE following treatment of FHV-1-infected CRFK cells with varying concentrations of raltegravir. (b, c) Raltegravir does not affect the epithelial layer thickness or viability. Paired corneas from the same cat were treated with ~2× the EC_{50} of raltegravir (500 µM) or left untreated and after 24 h, samples were collected for analyses. (b) Representative pictures of H&E-stained untreated and raltegravir-treated feline cornea sections and quantification of epithelial layer thickness. Bars, 300 µm. (c) Representative pictures of TUNEL-stained untreated and raltegravir-treated feline corneas. A mock-infected section was treated with DNase as a positive control. Bars, 100 µm. Results are presented as mean ±standard deviation; ns, not significant.
higher therapeutic index. For example, the integrase inhibitor XZ45 was shown recently to have an EC₅₀ as low as 1 µM against HSV-1 (Yan et al., 2014), and so it is planned in future experiments to test this integrase inhibitor in our feline corneal explant system.

Taken together, our current study is the first to report, to our knowledge, on the use of a whole corneal explant system to evaluate antiviral drugs to treat ocular FHV-1 infection, helping to bridge the gap between evaluating drugs in traditional cell culture and animals, and highlighting the importance of screening potential compounds in explant models prior to animal studies.

**METHODS**

**Virus, cells and antiviral drugs.** The FHV-1 strain FH2CS, isolated from a litter of kittens presenting to the New York State Veterinary College of Cornell University with signs of respiratory infection (Walton & Gillespie, 1970), was used in this study. Crandell Reese Feline Kidney (CRFK) cells were maintained in cell line media consisting of Dulbecco’s minimal essential medium (DMEM) with 1 g l⁻¹ glucose, l-glutamine and sodium pyruvate, 10 % foetal bovine serum (FBS), and penicillin (200 U ml⁻¹)/streptomycin (200 µg ml⁻¹), at 37 °C and 5 % CO₂. Acyclovir (EMD Millipore) and raltegravir (ChemieTek) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. Cidofovir (Gilead Sciences) was available as an intravenous solution (Vistide) and stored at room temperature.

**Collection of feline eyes.** Eyes were obtained from cats euthanized for reasons unrelated to this study (Liberty Research, Waverly, NY). These cats were raised under specific pathogen-free conditions and vaccinated against FHV-1, but were never exposed to an active infection. Random serological testing was performed monthly to ensure cats were not exposed to the virus. All cats sampled had not received boosters for at least four weeks prior to euthanasia. The eyes were assessed visually for abnormalities such as corneal abrasions or ulcers, anisocoria, ocular discharge, or strabismus by a trained Licensed Veterinary Technician. Cats were sedated with an intramuscular injection of ketamine and euthanized with either Euthasol (Virbac) or Beuthansia-D (Schering-Plough) via intracardiac injection. Whole eyes were removed immediately after euthanasia. Eyes were transported to the laboratory in sterile phosphate-buffered saline (PBS) containing a 2 % anti-microbial/anti-mycotic solution on ice.

**Fig. 5.** Incubation of FHV1-infected corneas with raltegravir reduces FHV-1 viral load and epithelial damage (n=3). Paired corneas from the same cat were both infected with 3×10⁵ PFU FHV-1 and were either left untreated or treated every 24 h with ~2× the EC₅₀ of raltegravir (500 µM). (a) Raltegravir significantly reduces FHV-1 replication in feline corneas. Viral load in the cornea was assessed by qPCR after 48 h, and produced infectious virus in the supernatants was assessed by standard plaque assays every 24 h. Dotted line represents the viral load in supernatants collected from FHV-1 infected, untreated matched corneas, set to 100 %. (b) Corneal cross-sections were stained with monoclonal anti-FHV-1 antibodies followed by Alexa Fluor 488-conjugated secondary antibodies, and nuclei were stained with DAPI. Bars, 100 µm. (c) Raltegravir reduces FHV-1-induced epithelial thinning in feline corneas. Cross-sections of FHV-1-infected, untreated and FHV-1-infected, raltegravir-treated corneas were stained with H&E and epithelial layer thickness was quantified. Bars, 300 µm. Results are presented as mean±standard deviation. **p<0.01; ****p<0.0001.
Whole corneal explant model system. Feline corneal explants were established as previously described for canine corneas, with some modifications (Harman et al., 2014). Clear corneas, including the limbus region and approximately 5 mm of the sclera, were aseptically and non-traumatically removed from the rest of the eye using a scalpel and dissection scissors [Fig. 1a(i)]. The iris and lens were removed and discarded [Fig. 1a(ii)]. Corneas were placed epithelial side down in a sterile ceramic plate (Avogadro’s Lab Supply), and the inner cavity formed by the cornea and sclera was filled with a 1% low-melting-point agarose solution in DMEM with 1 g l⁻¹ glucose, 1% glutamine and sodium pyruvate to provide structural support and maintain the normal three-dimensional shape of the cornea [Fig. 1a(iii)]. Corneas were turned epithelial side up, unless indicated otherwise, and further cultured in 12-well plates overlaid with cornea culture medium, consisting of DMEM, 10% FBS, penicillin (200 U ml⁻¹)/streptomycin (200 µg ml⁻¹), 1% nonessential amino acids, 1% sodium pyruvate, and 300 µg ml⁻¹ l-glutamine [Fig. 1a(iv)]. Corneas were used immediately for experiments and total time from eye collection to culture was approximately 1.5 h.

To evaluate the cytotoxicity of the antiviral drugs cidofovir, acyclovir and raltegravir, paired corneas from the same cat (n=1) were placed epithelial side down in 12-well culture plates and infected with 3×10⁹ PFU FHV-1 for 2 h at either 34°C or at 37°C, as described by Alekseev et al. (2012). Corneas were rinsed with PBS, placed epithelial side up in new 12-well plates containing 2.5 ml cornea culture media, and then cultured for an additional 24 h at either 34°C or at 37°C. Media was then collected and centrifuged at 10 000 g for 10 min to pellet cellular debris and these cell-free supernatants were frozen for viral titrations. The sclera was removed from the cornea and discarded. Corneas, epithelial side up, were bisected evenly in half, and snap-frozen for DNA extraction and PCR evaluation.

To evaluate the efficacy of the antiviral drugs cidofovir, acyclovir and raltegravir, paired corneas from the same cat were placed epithelial side down in 12-well culture plates and infected with 3×10⁹ PFU FHV-1 for 2 h at 34°C. Corneas were rinsed with PBS and then placed epithelial side up in new 12-well plates containing 2.5 ml cornea culture medium with 0 µM (control) or approximately twice the EC₅₀ of the drug (determined as described below), and cultured for 48 h. Media was collected and replaced with fresh media and drug every 2 or 24 h for the nucleoside analogues or raltegravir, respectively. Collected media were centrifuged as described above. At 48 h, the sclera was removed and discarded. Corneas were placed epithelial side up and bisected evenly in half, and snap-frozen for DNA extraction and PCR or embedded in clear frozen section compound (VWR) for histology and immunofluorescence analyses.

To evaluate the cytopathicity of raltegravir for feline corneas, paired corneas from the same cat were incubated epithelial side up in 12-well culture plates with 2.5 ml cornea culture medium containing 0 (control) or 500 µM raltegravir for 24 h at 34°C. Corneas were collected and embedded in clear frozen section compound for histology and immunofluorescence analyses.

Traditional two-dimensional CRFK cell cultures. The EC₅₀ of all antiviral drugs used in this study was determined, exactly as previously described, and was calculated based on the percent of wells with cytopathic effect (CPE) at each concentration (Ledbetter et al., 2015).

To evaluate cytopathicity of raltegravir for CRFK cells, the cells were grown to confluency in T25 flasks. Confluent monolayers were treated in triplicate with media containing increasing concentrations of raltegravir or a volume-matched amount of DMSO (control) and cultured for 24 h. Cells were treated with accutase, stained with 7-AAD (ThermoFisher Scientific) and assessed for viability via flow cytometry using a Gallios flow cytometer (Beckman Coulter) controlled by Kaluza for Gallios (version 1.0.14029.14028). Paraformaldehyde-fixed cells were included as a positive control for cell death. Data were analysed using Kaluza Analysis (version 1.3.14026.13330).

To evaluate the efficacy of raltegravir against FHV-1 replication in CRFK cells, three sets of experiments were performed. First, CRFK cells were plated at a density of 75 000 cells per well in 24-well plates, grown overnight, and infected with 75 PFU FHV-1 for 2 h at 37°C. Virus supernatants were removed and replaced with 0.94% carboxymethylcellulose in minimal essential medium (MEM), supplemented with 225 mg ml⁻¹ NaHCO₃, 10% FBS and penicillin/streptomycin, and containing increasing concentrations of raltegravir. Wells were cultured until viral cytopathic effect (CPE) in the controls was visible (~2 days). Mean plaque numbers per well were counted and the mean plaque size was measured using ImageJ (version 1.48). In the second experiment, confluent cells were infected for 2 h with 200 PFU FHV-1 in T25 flasks. Supernatants were removed and replaced with media containing 0 (control) or 500 µM raltegravir. Cell-free supernatants and cells were collected 24 h later for plaque assay analysis. In the third set of experiments, confluent CRFKs were infected for 2 h with 50 PFU FHV-1 in T25 flasks, and treated with 500 µM raltegravir at 0, 24 and 48 h post-infection (hpi). Twenty-four hours post drug treatment, cell-free supernatants and cells were collected for analysis.

Histology. Frozen corneas were cut into twelve 6 µm nonconsecutive sections and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) and imaged using the Aperio ScanScope. Mean epithelial measurements were quantified using the Aperio ImageScope software (version 12.1.0.0.0529) based on measurements taken approximately every 150 µm across the entire section. Representative images showing the average thickness of the epithelium were then compiled. Three separate, non-matched, mock-infected corneas cultured for 24 h were also included as controls.

Immunofluorescence (IF). An In Situ Cell Death Detection Kit (Fluorescein, Roche) was used, according to the manufacturer’s instructions, on cornea sections to analyse corneal cell viability (TUNEL assay). To ensure results were not due to technical error, sections of a control cornea, incubated with 1 mg ml⁻¹ DNase I (Sigma-Aldrich) in 50 mM Tris-HCL, pH 7.5, 10 mM MgCl₂ and 1 mg ml⁻¹ bovine serum albumin (BSA) for 1 h at 37°C, were included.

To detect FHV-1 by IF, a mouse monoclonal anti-FHV-1 (clone FHV7C; AbD Serotec) or isotype control antibody (Abcam), diluted 1:400 in PBS, was added to 2:3 (v/v) acetonitrile/25% acetic acid for 1 h. An Alexa-Fluor 488-conjugated secondary goat anti-mouse antibody (Jackson ImmunoResearch), diluted 1:100, was then added for 30 min. Nuclei were counterstained with 0.5 µg ml⁻¹ DAPI (EMD Chemicals Inc) and slides were fixed using Glycerel mounting medium (Dako). Five nonconsecutive cross-sections were analysed. Images were captured using a Zeiss LSM confocal microscope with an attached camera controlled by ZEN imaging software. Separate, non-matched, mock-infected corneal tissues were included as negative controls.

Viral plaque assays. To evaluate the release of infectious virus, extracellular virus titres were determined using standard plaque assays on CRFK cells, as previously described (Groth et al., 2014). Briefly, five sequential tenfold dilutions of the cell-free supernatant from either the two-dimensional or cornea assays were added onto monolayers of CRFK cells in 24-well plates to calculate PFU ml⁻¹.

Quantitative polymerase chain reaction (qPCR). To evaluate FHV-1 replication in CRFK or corneal cells, viral DNA copies were measured using qPCR. DNA from CRFK cell pelts or half of the corneas, as described above, was isolated using a DNeasy Blood & Tissue Kit (Qiagen). Primers used to detect viral and reference genes are listed in Table 1, and 30 ng DNA was added to triplicate wells of 96-well reaction plates with SYBR green master mix and 0.3 µM forward and reserve

http://jgm.microbiologyresearch.org/1423
primers. qPCR was performed using an ABI 7500 Fast Real Time PCR System (ThermoFisher Scientific). The comparative \( \Delta \Delta CT \) method (\( 2^{-\Delta \Delta CT} \)) was used to quantify gene expression levels. Reference values set to 100 consisted of DNA from either mock-infected or FHV-1-infected, untreated samples, and were used to calculate results as DNA fold change.

**Statistical analysis.** Data were statistically evaluated by GraphPad Prism (version 6.04 for Windows) and are expressed as the mean±standard deviation of triplicate independent experiments. For all analyses, \( p \leq 0.05 \) was considered significant.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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**Table 1.** Primers used in this study for quantitative PCR (qPCR)

<table>
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<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicon length (bp)</th>
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<tr>
<td>FHV-1 Glycoprotein E (gE)</td>
<td>F: GTGTTCACATCTCTACACCCG</td>
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<tr>
<td></td>
<td>R: CTCATGGCGGGTATATATCCGG</td>
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<tr>
<td>Infected Cell Polypeptide 4 (ICP4)</td>
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<tr>
<td></td>
<td>R: CTTCAATTCTGCATCGTGTTG</td>
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<tr>
<td>Cellular</td>
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<tr>
<td>Beta-2 microglobulin (B2M)</td>
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<tr>
<td></td>
<td>R: CAAGAGATCCAGCTTCACAGAGG</td>
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<tr>
<td>Ribosomal Protein L17 (RPL17)</td>
<td>F: AAGAACACACGGGAAACTGC</td>
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<tr>
<td></td>
<td>R: CTGGGCACACCTACCTACACTC</td>
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</table>

*F*, Forward; *R*, reverse.
replication in ocular and respiratory mucosae, the primary targets of infection. *Virus Res* 210, 227–231.


