In vitro cytotoxicity and antiviral efficacy against feline herpesvirus type 1 of famciclovir and its metabolites

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Abstract

Objectives To assess *in vitro* the antiviral efficacy against feline herpesvirus (FHV-1) and cytotoxicity for cultured feline cells of famciclovir and its metabolites, BRL 42359 and penciclovir. To investigate the effect of timing of penciclovir application on *in vitro* antiviral activity.

Procedures Plaque reduction assays were used to estimate antiviral efficacy of all compounds and the effect of penciclovir exposure before or after exposure to a FHV-1 field isolate. Cytotoxicity was evaluated by assessing cell morphology and viable cell number for 72 h following exposure to each compound.

Results The penciclovir concentration that inhibited FHV-1-induced plaque formation by 50% (IC_{50}) was 0.86 μg/mL (3.4 μM). Famciclovir and BRL 42359 had no antiviral effect against FHV-1 at any concentration assessed. Antiviral activity was significantly enhanced when cells were exposed to 4 μM penciclovir (approximate IC_{50}) for 1 h but not for 24 h before viral adsorption. Delaying exposure of cells to penciclovir for 1, 2, or 4 h after viral adsorption significantly enhanced antiviral activity. Relative to untreated control wells, >88% of cells remained viable when exposed to famciclovir (100 μM), BRL 42359 (1.06 mM), or penciclovir (40 μM) for 72 h. No morphologic evidence of cytotoxicity was noted.

Conclusions Penciclovir demonstrates potent antiviral activity against FHV-1 and may be effective at lower tissue, tear, and plasma concentrations than previously targeted. The duration of *in vitro* antiviral effect of penciclovir suggests that frequent famciclovir administration may be necessary *in vivo*. Famciclovir and BRL 42359 showed no signs of *in vitro* cytotoxicity.

Key Words: antiviral drugs, cat, herpetic disease, penciclovir, virology

INTRODUCTION

Infection of cats with feline herpesvirus type 1 (FHV-1) is very common and can result in ocular, respiratory, or dermatologic syndromes, some of which become chronic or recurrent and are difficult to treat. The use of antiviral agents to manage similar syndromes in herpes simplex virus type I (HSV-1)-infected humans is well established, and many drugs used to treat these conditions in humans have been studied for their potential role in the management of FHV-1 infections in cats. Although some of these medications had promising antiviral effects *in vitro*,[1–7] results of *in vivo* studies have sometimes been less encouraging.[8,9] In fact, some drugs marketed for the treatment of HSV-infected humans have proven poorly bioavailable, ineffective, or unsafe when used in FHV-1-infected cats.[8,9] By contrast, penciclovir is efficacious against FHV-1 *in vitro*[1–3,6] and, its oral prodrug – famciclovir – appears safe and effective when administered to FHV-1-infected cats.[10–13]

Famciclovir was formulated to overcome poor bioavailability of penciclovir following oral administration in humans.[14] Following oral administration, famciclovir is di-deacetylated in the plasma or small intestine to become 6-deoxypenciclovir (BRL 42359) which is then oxidized to penciclovir by a hepatic aldehyde oxidase.[15,16] Once penciclovir enters a virally infected cell, it is phosphorylated to

its active triphosphate form. The first phosphorylation step is catalyzed by a virally encoded thymidine kinase; the latter two steps are catalyzed by a host enzyme.[2,15–17] Currently available data suggest that the pharmacokinetics of famciclovir and penciclovir are especially complex in cats,[11,13,18] likely due to the virtual absence of hepatic aldehyde oxidase activity in this species.[19,20] Thus, cats administered relatively high doses of famciclovir achieve lower than expected plasma penciclovir concentrations. For example, cats receiving 90 mg famciclovir/kg orally three times daily[11] achieved a maximum median plasma penciclovir concentration of only 2.1 μg/mL (8.3 μM). This was notably lower than the target penciclovir concentration of 3.5 μg/mL (13.9 μM) based on the concentration of drug required to inhibit viral-induced plaque formation *in vitro* by 50% (IC_{50}) published at that time.[3,6] Despite this, cats experimentally infected with FHV-1 and receiving this dose of famciclovir had significantly improved outcomes for systemic, ophthalmic, clinicopathologic, virologic, serologic, and histologic variables when compared with placebo-treated cats.[11] In a subsequent open-label, non-placebo-controlled study,[10] cats receiving famciclovir at substantially lower and less frequent doses than those used in the experimental study[11] also showed marked clinical improvement; however, plasma penciclovir concentrations were not measured. Since the first reported IC_{50} for penciclovir against FHV-1 (13.9 μM),[3] other authors have reported values ranging from 1.2 to 130 μM.[1,2,6] Thus, reported *in vitro* efficacy of penciclovir ranges widely,[1–3,6] and clinical outcomes do not appear to necessarily correlate with plasma penciclovir concentrations.[10,11,13] There are a number of potential explanations for these observations. For example, it is possible that previously calculated IC_{50}s are excessive. Alternatively, it is possible that any of famciclovir, BRL 42359, or penciclovir exerts antiviral effects via mechanisms not predicted to date by *in vitro* testing. Third, it is possible that penciclovir or one of its phosphorylated forms accumulates intracellularly in feline cells, as has been demonstrated to occur in human lung fibroblast cells infected with HSV-1, HSV-2, and varicella-zoster virus.[21] Finally, *in vitro* testing of antiviral drugs is classically performed immediately after cultured cells are exposed to the virus, which does not accurately mimic clinical situations in which established and sometimes chronic viral infections are treated. Therefore, this study was designed to assess the *in vitro* antiviral efficacy of famciclovir, BRL 42359, and penciclovir and to investigate the effects of *in vitro* timing of penciclovir exposure relative to viral adsorption. Additionally, we assessed the *in vitro* cytotoxicity of famciclovir, BRL 42359, and penciclovir so as to confirm that any plaque reduction noted was not due to toxicity against the cell line chosen and so as to permit preliminary *in vitro* estimates of potential *in vivo* toxicity because these three compounds may accumulate in cats due to their unusual metabolism in this species.

MATERIALS AND METHODS

Virus, cells, and drugs

All experiments utilized the eighth passage of a plaque-purified field isolate of FHV-1 (727) that had been verified as FHV-1 with an immunofluorescent assay using antiserum specific for FHV-1.[22] This FHV-1 isolate has also been verified free of contamination with *Mycoplasma* spp., *Chlamydia felis*, and feline calicivirus by the use of PCR assays conducted at commercial testing laboratories (Specialized Infectious Disease Laboratory, Colorado State University, Fort Collins, CO and Lucy Whittier Molecular & Diagnostic Core Facility University of California-Davis, Davis, CA). Feline herpesvirus was cultured on Crandell Rees feline kidney (CRFK) cells. Penciclovir (9-[4-hydroxy-3-hydroxymethyl-but-1-yl]guanine) (Calbiochem, La Jolla, CA) was solubilized in phosphate-buffered saline (PBS) through adjustment of pH to 10.2 using NaOH. For the preparation of famciclovir, commercially available famciclovir tablets (250 mg tablets; Teva Pharmaceuticals, Sellersville, PA) were allowed to completely dissolve in tetrahydrofuran to form a 16 mg/mL solution. The solution was filtered, and the clarified solution was concentrated to a solid. The solid was dissolved in water and purified by high-performance liquid chromatography. A 16 mg/mL (50 mM) sterile solution of famciclovir was prepared in a laminar flow hood by dissolving 250 mg of isolated famciclovir solid into 15.4 mL sterile water for injection and passing the solution through a 0.22-μm filter. BRL 42359 was synthesized by deacetylation of famciclovir as described.[23] Briefly, an 80 mg/mL (250 mM) solution of isolated famciclovir in a 1:5 solution of 1 M hydrochloric acid/tetrahydrofuran was stirred for 20 h. Completion of the deacetylation reaction was observed by thin-layer chromatography, where plates of reaction solution developed in 2% methanol in dichloromethane showed disappearance of starting material (R_f = 0.404) and appearance of product (R_f = 0.11). The reaction solution was neutralized with sodium bicarbonate powder and concentrated to crude solid. Solid was dissolved in water and purified by high-performance liquid chromatography. Analysis by liquid chromatography–mass spectrometry confirmed the desired product. A 25 mg/mL (100 mM) sterile solution of BRL 42359 was prepared in a laminar flow hood by dissolving 185 mg of isolated solid into 7.4 mL of sterile water for injection and passing the solution through a 0.22-μm filter.

In vitro *antiviral efficacy of famciclovir, BRL 42359, and penciclovir*

To determine the antiviral efficacy of famciclovir, BRL 42359, and penciclovir against FHV-1 in CRFK cells, standard plaque reduction assays were performed as described.[3] Briefly, CRFK cells were cultured in a growth medium consisting of Dulbecco's modified Eagle medium

(DMEM) and 10% fetal bovine serum (FBS) in 12-well culture plates at 37 °C in 5% CO_2 at a seed intensity of 1×10^5 cells per well for 48 h, when they were approximately 80% confluent. Growth medium was gently aspirated from each well, and approximately 100 plaque-forming units of FHV-1 diluted in DMEM (approximate multiplicity of infection = 0.0001) were permitted to adsorb for 1 h at 37 °C in 5% CO_2 with gentle rocking at 15-min intervals. The FHV-1-containing DMEM solution was then gently aspirated, wells were gently washed with 1 mL DMEM at 37 °C, and CRFK cells were overlaid with carboxymethylcellulose solution alone (control) or containing one of multiple drug concentrations assessed (famciclovir: 5, 6.25, 12.5, 50, 63, 83, 125, 250, and 500 μM; BRL 42359: 5, 9, 13, 27, 52, 100, 157, 250, 500, and 1000 μM; and penciclovir: 1, 2, 4, 6, 8, 10, and 12 μM). The range of penciclovir concentrations tested was chosen based on previous studies.[1–3,6] Because reports of IC_{50} against FHV-1 were not available for famciclovir or BRL 42359, a wider range of concentrations was tested for these compounds. Each drug concentration was evaluated in triplicate with two replicates per experiment. For each drug concentration, the number of plaques from duplicate wells was counted and the mean value calculated. Penciclovir IC_{50} was calculated by plotting plaque reduction (%) against log penciclovir concentration. Linear regression was used to calculate a line of best fit, and the resulting equation was used to calculate the penciclovir concentration giving 50% plaque reduction (IC_{50}). The line of best fit was not forced through the zero intercept of the x- and y-axes.

Effect of timing of penciclovir application

A series of experiments was conducted to assess the effects of timing of penciclovir exposure relative to viral adsorption and to indirectly estimate whether penciclovir accumulates within CRFK cells. For all experiments in this series, penciclovir was added so as to achieve a final concentration of 4 μM, which approximated the IC_{50} calculated in the antiviral efficacy experiments described above. In the first set of experiments in the series, uninfected CRFK cells were exposed to 4 μM penciclovir for 1 or 24 h prior to viral adsorption. Following the prescribed period of penciclovir exposure, cells were gently rinsed using DMEM at 37 °C, and FHV-1 was permitted to adsorb for 1 h at 37 °C in 5% CO_2 with gentle rocking at 15-min intervals. Following viral adsorption, DMEM was aspirated from all wells, and CRFK cells were gently washed with 1 mL DMEM at 37 °C before being overlaid with carboxymethylcellulose solution without penciclovir. In the second set of experiments, exposure of CRFK cells to penciclovir was delayed for 1, 2, or 4 h after viral adsorption as follows. Following viral adsorption performed as detailed in the first set of experiments, infected CRFK cells were rinsed with DMEM and overlaid with 500 μL of drug-free DMEM with 1% fetal calf serum at

37 °C. Then, beginning at 0 (standard plaque reduction assay), 1, or 2 h after viral adsorption, penciclovir in DMEM was added to treatment wells so as to achieve a final concentration of 4 μM. Regardless of time of drug exposure, drug-containing media was aspirated 4 h following viral adsorption and cells were overlaid with carboxymethylcellulose solution without (control) or with 4 μM penciclovir. Thus, infected CRFK cells were exposed to 4 μM penciclovir 1, 2, or 4 h after viral adsorption. For both sets of experiments, plaque counts were assessed using 12-well plates, with three treatment wells and three untreated control wells per plate. In the first set of experiments, the number of viral plaques in treatment wells (cells exposed to penciclovir for 1 or 24 h before viral adsorption only) was compared to those in which penciclovir was never applied. For the second set of experiments, the number of viral plaques in treatment wells (cells in which penciclovir application was delayed by 1, 2, or 4 h after viral adsorption) was compared to those in which penciclovir was applied immediately after viral adsorption (standard plaque reduction assay). The number of plaques was compared among treatment protocols using a mixed effects linear regression model designed to analyze the effect of timing category (i.e., the fixed effect) on plaque count while using plate and well as random effects. Pairwise comparisons were adjusted using a Bonferroni correction. For all analyses, a P value ≤ 0.05 was considered significant.

In vitro *cytotoxicity of famciclovir, BRL 42359, and penciclovir*

Cytotoxicity assays were performed on uninfected CRFK cells cultured in 6-well plates containing DMEM with 10% FBS and drug-free diluent (control) or famciclovir (100 μM), BRL 42359 (1.06 mM), or penciclovir (40 μM). This penciclovir concentration was chosen because it represented approximately 10 × IC_{50}. Because an IC_{50} could not be calculated for BRL 42359 and famciclovir, maximal concentrations of these drugs that could be placed into solution were chosen. After 24, 48, and 72 h of incubation, cells from one control well and one well containing each drug concentration were examined by the use of an inverted microscope to detect morphologic changes and to evaluate confluence. Cells then were harvested by enzymatic detachment with trypsin–EDTA solution. Following complete detachment of all cells from the wells, trypsin was deactivated by the addition of growth media, cell-containing media was centrifuged at 300 g for 6 min, and the supernatant was carefully aspirated and discarded. The cellular pellet was resuspended in a known volume of DMEM, stained using a known volume of trypan blue, and viable cells were counted on a hemocytometer. All experiments were performed in duplicate, and viable cell number was counted twice. Total number of viable cells was calculated for each well, and viable cell numbers for each drug concentration were expressed as

the percentage reduction relative to control wells containing no drug.

RESULTS

In vitro *antiviral efficacy of famciclovir, BRL 42359, and penciclovir*

Using standard plaque reduction assay methodology (i.e., addition of the test compound immediately following viral adsorption only) neither famciclovir nor BRL 42359 showed appreciable antiviral effect against FHV-1 at any concentrations assessed (Fig. 1). By contrast, penciclovir had notable antiviral activity, with a calculated IC_{50} of 3.4 μM.

Effect of timing of penciclovir application

Exposure of CRFK cells to 4 μM penciclovir (approximate IC_{50}) for 1 h prior to viral adsorption was associated with significantly reduced ($P = 0.016$) mean ± SEM viral plaque count (52.4 ± 4.6) relative to that when penciclovir was not applied prior to viral adsorption (59.89 ± 4.4; Fig. 2). However, no significant difference ($P = 0.630$) in mean ± SEM viral plaque count was detected between wells exposed to penciclovir for 24 h before viral adsorption only (61.89 ± 5) and wells that were not exposed to penciclovir at any time (59.89 ± 4.4; Fig. 2). Delaying exposure of CRFK cells to 4 μM penciclovir for 1, 2, or 4 h after viral adsorption was associated with significantly reduced ($P < 0.001$) mean ± SEM viral plaque count (24.3 ± 3.4, 23.8 ± 3.2, or 31.7 ± 3.2, respectively) relative to that seen with the standard plaque reduction assay (62.8 ± 3.2; Fig. 3).

Figure 2. Effect of exposure of Crandell Rees feline kidney (CRFK) cells to penciclovir for various durations prior to viral infection. Uninfected CRFK cells were exposed to 4 μM penciclovir (approximate IC_{50}) for 1 or 24 h before viral adsorption and then gently rinsed. Control cells had no exposure to penciclovir. Antiviral activity was significantly enhanced relative to control by exposure to penciclovir for 1 h ($P = 0.016$)* but not 24 h ($P = 0.630$) prior to viral adsorption.

In vitro *cytotoxicity of famciclovir, BRL 42359, and penciclovir*

Relative to untreated control wells, >88% of CRFK cells remained viable when exposed to famciclovir, BRL 42359, or penciclovir at the concentrations tested for 24, 48, or
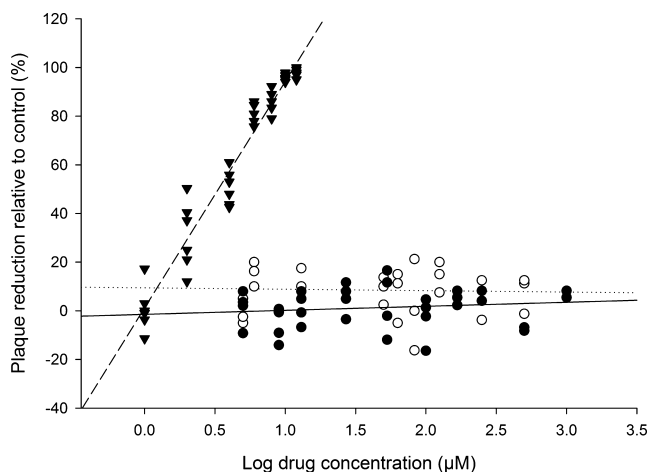

Figure 1. Antiviral effect (expressed as percentage plaque reduction relative to untreated control) of multiple concentrations of famciclovir (open circles), BRL 42359 (filled circles), and penciclovir (filled triangles) against feline herpesvirus type 1 cultured on Crandell Rees feline kidney cells. Regression equations and r^2 coefficients are famciclovir: $y = -0.6x + 9.4$, $r^2 = 0.00$ (dotted line); BRL 42359: $y = 1.7x -1.5$, $r^2 = 0.02$ (solid line); penciclovir: $y = 93.1x + 1.2$, $r^2 = 0.95$ (dashed line).
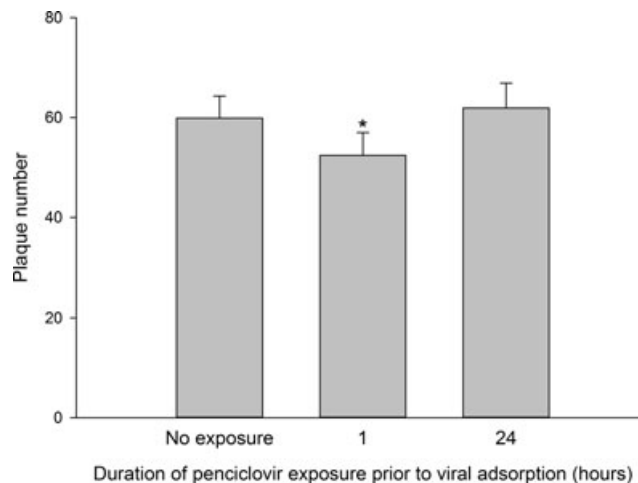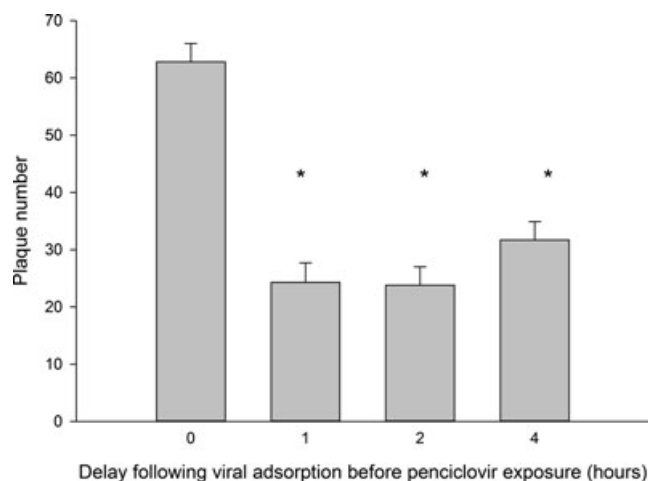

Figure 3. Effect of exposure of Crandell Rees feline kidney (CRFK) cells to penciclovir at various delays after viral adsorption. Infected CRFK cells were exposed to 4 μM penciclovir (approximate IC_{50}) following a delay of 1, 2, or 4 h after viral adsorption. The number of viral plaques produced when penciclovir exposure was delayed by 1, 2, or 4 h following adsorption was compared with the number of viral plaques produced when there was no delay (0 h). *Delaying exposure of CRFK cells to penciclovir by 1, 2, or 4 h after viral adsorption resulted in significantly enhanced ($P < 0.001$ for each) antiviral activity relative to that achieved when penciclovir exposure was not delayed (0 h).

Table 1. Cytotoxicity of penciclovir (40 μM), famciclovir (100 μM), and BRL 42359 (1060 μM) was assessed after 24, 48, and 72 h of exposure of Crandell Rees feline kidney (CRFK) cells to each compound. Data are presented as percentage number of viable CRFK cells relative to drug-free diluent (control) at each time point. No morphologic changes were noted

Duration of exposure of CRFK cells to each compound	Number of viable CRFK cells relative to drug-free diluent (control)		
	Penciclovir, 40 μM (%)	Famciclovir, 100 μM (%)	BRL 42359, 1060 μM (%)
24 h	88.4	95	111.5
48 h	86.8	126	107.5
72 h	90.3	126	101.5

72 h (Table 1). No morphologic evidence of cytotoxicity was noted for any compound, at any of the time points assessed.

DISCUSSION

Data from the present study reveal that neither the prodrug famciclovir nor the intermediate metabolite BRL 42359 (6-deoxypenciclovir) has an appreciable antiviral effect against FHV-1 at the concentrations assessed, but that penciclovir is a highly efficacious antiviral agent against FHV-1. The IC$_{50}$ value for penciclovir determined in the present study (0.86 μg/mL or 3.4 μM) is toward the lower end of the range of estimated IC$_{50}$s published for penciclovir (1.2–130 μM).[1–3,6] Numerous differences in methodology among studies likely explain in part this wide variation in published IC$_{50}$s, but also make comparison of data among studies difficult. Some differences in methodology among studies include the multiplicity of infection (number of viral particles applied per cell *in vitro*) used for the plaque reduction assays,[2] viral strain used and, in particular, its resistance to penciclovir,[1] assessment of plaque number vs. plaque size as a measured outcome,[5] and the interval between inoculation and plaque assessment. In the present study, virus strain, multiplicity of infection, and duration of viral adsorption and replication were kept constant among all replicates for all drugs. Plaque number was also consistently assessed among replicates; plaque size was not considered. The method of data analysis also varies among studies and, in some studies, is not clearly described, and yet, it appears that this is very important.[24] For example, it is not clear when calculating the line of best fit (Fig. 1) whether a straight line or more complex equation is more appropriate, nor whether this line should be 'forced' to pass through the zero intercept.[3,5–7] In the present study, a straight line without a forced zero intercept was used. Controlling for and reporting these variables in future studies are recommended and would likely result in improved agreement and easier comparison of IC$_{50}$ estimations among studies. The reason for the different estimate of IC$_{50}$ in the present and previous[3] studies both conducted in our laboratory using similar methodology and the same viral strain cannot be determined with certainty but is likely due to solubility of penciclovir. In the previous study,[3] the stock solution was made by dissolving penciclovir in PBS with a pH of 7.4. This stock solution was then stored at 4 °C for the course of the experiments and, retrospectively, the penciclovir is believed to have come partially out of solution during that period. In the present study, we found that penciclovir was inadequately dissolved at this temperature and pH and so adjusted the pH to 10.2 using NaOH.

To the authors' knowledge, this is the first report investigating the effect of timing of penciclovir exposure relative to viral infection on antiviral efficacy of this drug against FHV-1. We assessed these effects of timing in two ways. In the first set of experiments, we examined whether pretreatment of CRFK cells with penciclovir prior to viral adsorption had a lasting effect when cells were subsequently inoculated. Our data revealed that there was a persistent antiviral effect *in vitro* when cells were exposed to penciclovir for 1 h but not for 24 h prior to viral adsorption. There are numerous potential explanations for these observations. It is possible that, after 1 h of exposure, penciclovir may have become unstable under the conditions tested or been metabolized by CRFK cells. Alternatively, it is possible that penciclovir exposure for >1 h was associated with some loss of cellular function that made CRFK cells more susceptible to viral infection despite penciclovir persisting in a functional form. However, this putative dysfunction was not associated with lack of cell viability sufficient to be detected in the subsequent cytotoxicity experiments reported here. Our second set of experiments assessed the effect on antiviral activity of delaying application of penciclovir for 1, 2, or 4 h following viral adsorption. For this set of experiments, the control wells underwent a standard plaque reduction assay in which penciclovir was applied immediately following viral adsorption. We showed that delaying penciclovir for up to 4 h after viral adsorption significantly increased the antiviral activity of this drug. This is likely explained by the fact that the first of three phosphorylation steps necessary for drug activation is mediated by a virally-encoded thymidine kinase enzyme.[2,17] Thus, allowing up to 4 h of viral replication likely permitted a greater concentration of viral thymidine kinase to accumulate prior to penciclovir application.

In vitro data generated in the present study must be cautiously extrapolated to clinical use of famciclovir in cats infected with FHV-1. However, they may aid in the interpretation of *in vivo* efficacy and pharmacokinetic data currently available regarding penciclovir and its oral prodrug, famciclovir. In the only study to date jointly assessing famciclovir pharmacokinetics and efficacy in cats,[11] we showed that famciclovir was highly effective when administered at 90 mg/kg three-times-daily to cats experimentally inoculated with FHV-1. However, peak

plasma concentration of penciclovir in those cats (approximately 2.1 μg/mL; 8.3 μM) was below the concentration targeted based upon the first reported IC_{50} for FHV-1 (3.5 μg/mL or 13.9 μM).[3] Results from the present study, along with those from other *in vitro* studies,[1,6] suggest that the IC_{50} calculated in our previous study[3] may represent an overestimation of the IC_{50} (i.e., an underestimate of antiviral activity) of penciclovir for FHV-1. Utilizing the IC_{50} calculated in the present study (3.4 μM), all approximate penciclovir peak concentrations (range, 4.8–22.9 μM) and some trough penciclovir concentrations (1.2–11.0 μM) in cats administered 90 mg/kg of famciclovir three times daily would have exceeded this new target concentration.[11] In addition, a single oral dose of 40 or 90 mg of famciclovir/kg or intravenous infusion of 10 mg/kg of penciclovir would maintain plasma penciclovir concentrations above this revised IC_{50} (3.4 μM) for at least 5 h.[13,18] It is also interesting to postulate how the *in vitro* data in the present study regarding timing of drug exposure relate to spontaneous infections in cats. It seems reasonable that, in naturally infected cats and especially those with low-grade recrudescent disease, host cells at all stages of viral replication from uninfected through cell lysis would be present in corneal, conjunctival, or dermal epithelium. Thus, dosing intervals and penciclovir clearance rates from these tissues would be important determinants of when individual epithelial cells in infected cats would be exposed to penciclovir relative to viral infection. Our data showing that penciclovir has no appreciable *in vitro* effect if present for 24 h prior to infection makes a rational argument for the administration of famciclovir sufficiently frequently to ensure exposure of infected cells to penciclovir more often than once every 24 h. The *in vitro* timing data presented here are particularly interesting when considered in association with evidence that tear penciclovir concentrations in cats receiving 39–72 mg famciclovir/kg three times daily exceeded the lowest published IC_{50} for FHV-1[1] for approximately 3 h following each dose.[18] This suggests that cats receiving famciclovir according to this regimen would have tear penciclovir concentrations below target for only three 5-h periods within every 24 h. However, in the present study, we have also shown that penciclovir has an appreciable antiviral effect if applied to cells within 4 h following viral exposure and that this effect lasts for at least 1 h after the penciclovir is withdrawn. If these *in vitro* data hold true *in vivo*, corneal and conjunctival cells of cats receiving famciclovir three times daily will be exposed to tear penciclovir concentrations expected to be effective against FHV-1 at all times other than three 1-h periods daily. Taken together, data presented here estimating duration of *in vitro* effect of penciclovir, along with previously published data regarding clinical efficacy of three-times-daily famciclovir dosing,[11] and pharmacokinetics of penciclovir in tears[18] lend further support to three-times-daily administration of 40 mg famciclovir/kg to cats infected with FHV-1.

To the authors' knowledge, this is the first report evaluating the toxicity for feline cells in culture of famciclovir and BRL 42359. Understanding the limitations of extrapolating *in vitro* data to *in vivo* conditions, this could have important clinical implications. The metabolism of famciclovir to penciclovir in cats is nonlinear; likely due to saturation of hepatic aldehyde oxidase, which is essential for the conversion of the inactive intermediate metabolite, BRL 42359, to penciclovir.[11,18,20] Because this enzyme is almost absent in cats,[19,20] and because absorption of famciclovir and conversion of famciclovir to BRL 42359 appear to be relatively rapid while conversion from BRL 42359 to penciclovir appears incomplete,[18] accumulation of BRL 42359 with consequent relatively high plasma concentrations of this compound is likely but has not been proven in cats. Likewise, a definitive dose rate of famciclovir for cats has not been established, and highly variable dose rates and dose frequency have been published, some as high as 90 mg/kg three times daily.[10,11] For all of these reasons, our preliminary assessment of the effects of these three compounds on the morphology and viability of CRFK cells in the present study is important. Famciclovir and BRL 42359 at the highest concentrations that could be dissolved and penciclovir at 10 times the IC_{50} (a concentration that has never been recorded in the plasma of cats receiving doses of famciclovir as high as 90 mg/kg three times daily) caused no appreciable morphologic or quantitative evidence of cytotoxicity.[11,13,18] These observations should be repeated using cultured lines of feline epithelial cells,[25–27] and more *in vivo* investigations are required in cats of various ages and health status. However, cytotoxicity data from the present study support data generated in a small number of experimental and client-owned cats which have received famciclovir to date,[10–13,18] all of which suggest minimal toxicity of famciclovir and penciclovir in cats.

Studies to date have suggested famciclovir is highly effective for treatment of experimental[11] and naturally occurring[10,12] herpetic disease in cats despite being shown[11] or likely[10] not to always achieve plasma penciclovir concentrations targeted at that time.[3] In the present study, we have demonstrated that direct antiviral efficacy of famciclovir or its metabolite, BRL 42359, is highly unlikely to explain this observation. Rather, data from the present study suggest that the most likely explanation for the greater than expected antiviral efficacy *in vivo* is that penciclovir exerts a more potent antiviral effect than demonstrated in many previous *in vitro* studies. Data presented here also suggest penciclovir may exert a greater antiviral effect in cells with established viral infection than those very recently infected.

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