In vitro efficacy of ganciclovir, cidofovir, penciclovir, foscarnet, idoxuridine, and acyclovir against feline herpesvirus type-1

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Objective—To establish the in vitro efficacy of 4 novel drugs (ie, ganciclovir, cidofovir, penciclovir, and foscarnet) against feline herpesvirus type-1 (FHV-1) and compare their antiviral efficacy with that of acyclovir and idoxuridine.

Sample Population—Cultured Crandell-Reese feline kidney (CRFK) cells and FHV-1 strain 727.

Procedure—For each drug, antiviral effect was estimated by use of conventional plaque-reduction assays, and inhibitory concentration 50 (IC50; drug concentration at which plaque numbers were reduced by 50% relative to the number of plaques for nontreated control wells) was calculated. To determine whether observed antiviral effects were related to alterations in the number or viability of CRFK cells, cytotoxicity assays were performed at 1, 2, and 10 times the median IC50 for each antiviral drug.

Results—Median IC50 for each drug was as follows: ganciclovir, 5.2µM; cidofovir, 11.0µM; penciclovir, 13.9µM; foscarnet, 232.9µM; idoxuridine, 4.3µM; and acyclovir, 57.9µM. Obvious changes in morphologic characteristics, confluence, or viability of CRFK cells were not observed at concentrations up to and including 2 times the IC50 for each drug.

Conclusions and Clinical Relevance—In vitro efficacy of idoxuridine and ganciclovir against FHV-1 was approximately equivalent and about twice that of cidofovir and penciclovir. Foscarnet appeared to be comparatively ineffective. Given the reasonable clinical efficacy of idoxuridine in cats infected with FHV-1, clinical trials of ganciclovir, cidofovir, and penciclovir or their prodrug forms appear to be warranted. (Am J Vet Res 2004;65:399–403)

Feline herpesvirus type-1 (FHV-1) and herpes simplex virus type-1 (HSV-1) are alphaherpesviruses that share a number of inherent biological characteristics, including a short reproductive cycle, rapid replication and spread in culture, efficient lysis of infected cells, and capacity to establish latency, especially in sensory ganglia. Both viruses also induce almost identical disease syndromes in their specific hosts. Primary FHV-1 infection commonly manifests within the first few months after birth, usually causing signs of self-limiting respiratory and ocular disease. Approximately 93% of adult cats are seropositive for FHV-1. Although primary infections are usually self-limiting, lifelong latency is established in the trigeminal ganglia of approximately 80% of cats. It is estimated that half of these cats later shed FHV-1 intermittently, sometimes without clinical signs. These periods of shedding can be induced by stresses such as pregnancy, lactation, or changes in housing or as a result of glucocorticoid administration. However, 29% of latently infected cats shed virus spontaneously. A small group of naturally infected cats may develop recurrent stromal keratitis, which is clinically, virologically, immunologically, and histopathologically almost identical to herpetic stromal keratitis in humans. Some cats are also affected by chronic rhinosinusitis, in which FHV-1 is suspected to play some role.

Because of the striking similarities between HSV-1 and FHV-1, drugs originally developed for the treatment of humans infected with herpesviruses are often used to treat cats infected with FHV-1. The efficacy against FHV-1 of some of these compounds has been tested and found to be as expected from data generated with HSV-1. In other cases, these investigations have unexpectedly highlighted poor efficacy or unacceptable toxic effects. Therefore, antiviral treatments developed for use in humans with herpetic infections require thorough in vitro and in vivo testing prior to their clinical application in cats.

The purpose of the study reported here was to establish in vitro efficacy against FHV-1 of 4 relatively new antiviral drugs (ganciclovir, cidofovir, penciclovir, and foscarnet) developed for the treatment of humans with herpetic infections. Additionally, we intended to compare the in vitro efficacy of these 4 drugs with that of 2 other antiviral drugs (acyclovir and idoxuridine) whose in vitro efficacy and mechanism of action have been studied and that are in fairly widespread clinical use as systemically (acyclovir) or topically (idoxuridine) administered medications in the treatment of cats infected with FHV-1.

Materials and Methods

Sample population—Crandell-Reese feline kidney (CRFK) cells and the eighth passage of a plaque-purified field isolate of FHV-1 were used for all viral cultures. Ganciclovir (9-[1,3-dihydroxy-2-propoxy-methyl]guanine), cidofovir (1-{[S]-3-hydroxy-2-phosphonomethoxy}propyl)cytosine), penciclovir (9-[4-hydroxy-3-hydroxymethyl-buty1-y1]guanine), foscarnet (phosphonoformic acid), idoxuridine (5-iodo-2-deoxyuridine), and acyclovir (9-[2-hydroxyethoxymethyl]guanine) were obtained from commercial

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Antiviral compounds were dissolved in PBS solution and stored at 4°C until use.

**Antiviral assays**—Standard plaque reduction assays were performed as described elsewhere. Briefly, CRFK cells were cultured in a growth medium consisting of Dulbecco modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS) in 12-well culture plates at 37°C in 5% CO₂ until approximately 75% to 95% confluency was reached. Growth medium was decanted from each well, and approximately 100 plaque-forming units of FHV-1 diluted in DMEM were permitted to adsorb for 1 hour at 37°C in 5% CO₂ with gentle rocking at 15-minute intervals. The FHV-1 and DMEM solution was then decanted, and cells were overlaid with carboxymethylcellulose solution alone or carboxymethylcellulose solution containing 1 of 5 concentrations of each drug. Each drug concentration and nontreated control solution were assessed in duplicate wells. Preliminary experiments were conducted for each drug by use of a range of concentrations (1 to 300 μM) to estimate the inhibitory concentration 50 (IC₅₀), defined as the drug concentration at which plaque numbers were reduced by 50% relative to the number of plaques for nontreated control wells. Five concentrations close to the IC₅₀ of each drug were then selected, and 3 to 5 replicates were performed for each concentration. Concentrations used and the number of replicates for each drug were as follows: ganciclovir (2.5, 5.0, 7.5, 10.0, and 12.5 μM; n = 3), cidofovir (3, 5, 10, 15, 20, and 25 μM; 5), penciclovir (3, 6, 9, 12, and 15 μM; 5), foscarnet (125, 150, 175, 200, and 225 μM; 4), idoxuridine (2, 4, 6, 8, and 10 μM; 4), and acyclovir (20, 40, 60, 80, and 100 μM; 3).

**Cytotoxicity assays**—To determine whether observed antiviral effects were related to alterations in the number or viability of CRFK cells, cytotoxicity assays were performed once for each of 3 concentrations for each drug used, and a computer-generated line of best fit and an equation describing the relationship were constructed. Median (range) r² value for these lines was 0.98 (0.86 to 1.00). The IC₅₀ was calculated for each replicate, and the median IC₅₀ was calculated for each drug. Antiviral efficacy was compared among drugs by use of an exact Mann-Whitney U test. Tests were conducted by use of commercially available statistical software. Values of P < 0.05 were considered significant.

**Results**

**Antiviral effect**—The median IC₅₀ of each antiviral drug was calculated (Table 1). In vitro efficacy (ie, median IC₅₀) of idoxuridine (4.3 μM) and ganciclovir (5.2 μM) was approximately equivalent (P = 0.40) but significantly (P = 0.016 and 0.036, respectively) superior to that of cidofovir (11.0 μM) and penciclovir (13.9 μM). In vitro efficacy of acyclovir (median IC₅₀, 57.9 μM) and foscarnet (232.9 μM) was significantly (P = 0.036 and 0.016, respectively) less than that of penciclovir.

**Cytotoxicity assays**—Obvious changes in CRFK cell morphology, confluence, or viability were not observed at concentrations 1 or 2 times the IC₅₀. However, at concentrations 10 times the IC₅₀, decreased confluence and, in some cases, altered morphologic characteristics were evident for idoxuridine, cidofovir, foscarnet, and acyclovir. The most commonly observed morphologic alteration was rounded cells, some of which were detached from the tissue flask, clumped, or both. For all drugs tested, the number of CRFK cells decreased with increasing duration of culture and increasing drug concentration (data not shown). For the 72-hour culture period, mean daily reduction in the number of uninfected CRFK cells cultured in medium containing drugs at 1 times the median IC₅₀ typically ranged from 5.5% to 12.3% (ganciclovir, cidofovir, foscarnet, acyclovir, and idoxuridine; Table 1). Greatest mean daily reduction in the number of CRFK cells during the 72-hour period (25%) was seen with penciclovir.

**Table 1**—Antiviral efficacy against feline herpesvirus type-1, expressed as median (range) values of the inhibitory concentration 50 (IC₅₀), and cytotoxic effects at median IC₅₀, expressed as the mean daily decrease in number of uninfected Crandell-Reese feline kidney (CRFK) cells during a 72-hour culture period, for 6 antiviral drugs

<table>
<thead>
<tr>
<th>Antiviral drug</th>
<th>Median (range) IC₅₀(μM)</th>
<th>Relative decrease in CRFK cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganciclovir</td>
<td>5.2 (4.8–5.4)</td>
<td>5.5</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>11.0 (10.0–11.6)</td>
<td>9.1</td>
</tr>
<tr>
<td>Penciclovir</td>
<td>13.9 (12.5–14.2)</td>
<td>25.0</td>
</tr>
<tr>
<td>Foscarnet</td>
<td>232.9 (178.1–253.8)</td>
<td>10.1</td>
</tr>
<tr>
<td>Idoxuridine</td>
<td>4.3 (3.7–5.2)</td>
<td>10.8</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>57.9 (55.8–67.69)</td>
<td>12.3</td>
</tr>
</tbody>
</table>

*The IC₅₀ is defined as the drug concentration at which plaque numbers were reduced by 50% relative to the number of plaques for nontreated control wells.
Discussion

At least 40 antiviral compounds have been licensed for clinical use or are in advanced clinical trials in humans. Of these, many have excellent efficacy against herpesviruses. However, antiviral efficacy against even closely related viruses, such as HSV-1 and FHV-1, is not predictable for any given drug. Therefore, the study reported here was conducted to examine the efficacy against FHV-1 of 4 relatively new antitherpetic drugs (ganciclovir, cidofovir, penciclovir, and foscarnet). Two drugs (idoxuridine and acyclovir) whose in vitro" and clinical efficacy against FHV-1 have been measured were also included to provide a frame of reference for the data reported here. The IC₅₀ for acyclovir (57.9µM) and idoxuridine (4.3µM) determined in our study is comparable to values reported by other authors. Analysis of our data suggests that in vitro efficacy of the 6 drugs tested (ranked on the basis of median IC₅₀) was as follows:

idoxuridine ≈ ganciclovir >> cidofovir ≈ penciclovir >> acyclovir = foscarnet.

This, along with data from clinical studies, suggests that ganciclovir, cidofovir, and penciclovir may have some clinical use in the treatment of cats infected with FHV-1. By contrast, foscarnet appears to be relatively ineffective against FHV-1.

Acyclovir, ganciclovir, and penciclovir (along with their relevant prodrug forms) are all acyclic nucleoside analogues. As a group, these agents share many properties. All require 3 phosphorylation steps for conversion to their active triphosphate forms. The first of these phosphorylation steps is catalyzed by a viral enzyme (typically a kinase); the subsequent 2 steps are catalyzed by host kinases. The primary, virally mediated phosphorylation step determines, in part, the specificity of these agents for virally infected cells and accounts for their relative safety, compared with that of many other antiviral agents. The first phosphorylation step is also a major factor in determining the susceptibility of specific viruses to these drugs. Certain kinase-deficient strains of herpesviruses, as well as many non-herpesviruses, are predictably resistant to the acyclic nucleoside analogues. Because FHV-1 and some other herpesviruses are relatively resistant to acyclovir, some authors have suggested that these viruses lack the kinase necessary to initiate activation of acyclovir. The relatively high IC₅₀ for acyclovir in the study reported here confirms the relative resistance of FHV-1 to acyclovir.

Because the acyclic nucleoside analogues share a similar requirement for enzymatic phosphorylation and activation, the relatively high antiviral efficacy we determined for ganciclovir (IC₅₀, 5.2µM) and penciclovir (IC₅₀, 13.9µM), compared with acyclovir (IC₅₀, 57.9µM), is noteworthy. There are numerous potential explanations for the apparent enhanced efficacy of ganciclovir and penciclovir, many of which have been documented for these and other related drugs with other viruses. These explanations include variations in the rate or extent of cellular uptake of each drug, rate or efficiency with which viral or cellular enzymes phosphorylate each drug, intracellular stability of each drug metabolite, and potency and mechanism by which these drugs inhibit viral DNA polymerases. For those herpesviruses affecting humans, all 3 acyclic nucleoside analogues act by substituting a deoxynucleoside triphosphate into the growing viral DNA; however, when acyclovir does this, it acts as an obligatory chain terminator, whereas penciclovir and ganciclovir can insert a deoxynucleoside triphosphate at intermediate sites as well as at chain termini.

The nature of the interaction between these 3 drugs and FHV-1 DNA polymerase or the susceptibility of this enzyme to these drugs is not known.

Regardless of the cause for the increased potency of ganciclovir and penciclovir relative to acyclovir, clinical trials in cats to determine the safety and efficacy of penciclovir and ganciclovir or their prodrug forms (famiclovir and valganciclovir respectively) appear justified on the basis of data reported here. Such studies must be conducted with great caution because the closely related drug, acyclovir, sometimes has toxic effects in cats. Increased bioavailability afforded by administering prodrug forms may also increase toxicosis, as has been determined for the acyclovir prodrug valacyclovir. Bioavailability of valacyclovir is more than 3-fold greater than acyclovir in humans and 2.3-fold greater in cats. Although administration of valacyclovir (60 mg/kg, PO, q 6 h) resulted in higher plasma concentrations of acyclovir in cats than can be achieved with similar doses of acyclovir, this dose of valacyclovir was also associated with severe bone marrow suppression and hepatic and renal necrosis, but no observable antiviral effect in cats infected with FHV-1. It is possible that the increased in vitro efficacy of ganciclovir and penciclovir relative to acyclovir may be associated with an increased therapeutic index. Increased bioavailability of the respective prodrug forms may then increase plasma concentrations into a therapeutic range without associated toxic effects.

In the study reported here, we were not able to document useful antiviral activity of foscarnet against FHV-1. The reason for this is not known. In contrast to the acyclic nucleosides, foscarnet does not require phosphorylation to achieve antiviral activity, thus, the necessity of the appropriate FHV-1 or host (feline) kinases or their affinity for foscarnet is unlikely to be the explanation. Rather, foscarnet directly inhibits viral DNA polymerase by interacting with the pyrophosphate binding site of that enzyme. The affinity of foscarnet for and its mode of action on FHV-1 DNA polymerase are unknown. Other virus strains resistant to foscarnet sometimes have mutations of their DNA polymerase or altered replication rates. We studied only 1 strain of FHV-1 in the study reported here, and although it is possible that there was a DNA polymerase mutation in this strain, other studies of FHV-1 homogeneity that used molecular techniques or in vitro drug trials have suggested that this is a relatively conserved virus throughout the world with remarkably uniform susceptibility to antiviral drugs.

For a number of reasons, cidofovir (a cytosine analogue) is 1 of the more encouraging drugs for the treatment of cats with herpesvirus infections. Analysis of our data revealed that this drug was highly effective at reducing in vitro replication of FHV-1. Its IC₅₀ was...
about double that for idoxuridine, which has proven to be reasonably effective in clinical use.\textsuperscript{11,12,24} In contrast to the acyclic nucleoside analogues, cidoflovir does not require initial viral phosphorylation, thus avoiding concerns regarding FHV-1 kinase affinity. However, cidoflovir does require 2 host-mediated phosphorylation steps for activation. Although both of these steps happen in uninfected cells, they are accentuated in virally infected cells.\textsuperscript{25,26} Additionally, cidoflovir inhibits viral DNA polymerase approximately 1,000 times more potently than it does host DNA polymerase.\textsuperscript{27} These factors greatly increase the therapeutic index of this drug and permit it to be administered systemically as well as topically.\textsuperscript{28} They are also the reason for its extremely broad antiviral spectrum, which includes most DNA viruses studied, including acyclic nucleoside analogue-resistant, enzyme-deficient strains.\textsuperscript{29} Cidoflovir has been invariably proven superior to acyclovir, trifluridine, penciclovir, and ganciclovir when tested in animals infected with human herpesviruses.\textsuperscript{30,31} Cidoflovir has been administered IV, intravitreally, or topically as a dermatologic or ophthalmic preparation in humans.\textsuperscript{32}

Perhaps of most interest for applications in cats is the ability to administer cidoflovir less frequently than other antiviral agents. This is possible because of its long-lasting antiviral effect, which is presumed to be attributable to the long half-life (up to 48 hours) of its metabolites. In vitro antiviral effects were evident for at least 7 days after only 6 hours of exposure to cidoflovir,\textsuperscript{33} whereas IV administration of cidoflovir as infrequently as once every 1 to 2 weeks has been recommended in human patients. Relatively infrequent topical application of this compound to the eyes of rabbits infected with HSV-1 has also yielded promising results.\textsuperscript{34} In that study, severity of keratitis and extent of viral shedding were significantly reduced in rabbits receiving cidoflovir only twice daily, compared with results for those receiving acyclovir 5 times daily or trifluridine 4 to 9 times daily. An efficacious antiviral drug that could be administered infrequently to cats with FHV-1 infection would be greatly advantageous, especially because stress should be reduced in cats with recrudescent herpetic disease.

Finally, potent synergy has been documented between cidoflovir and lactoferrin (a glycoprotein found in mammalian secretions, including tears). Suppression of in vitro replication of a human herpesvirus (ie, cytomegalovirus) was 42% greater when cidoflovir was combined with lactoferrin than when either compound was used alone.\textsuperscript{35} In contrast, an antagonistic effect was evident for the combination of lactoferrin and acyclovir or foscarnet, and no interactive effect was evident for the combination of lactoferrin and ganciclovir. In another study,\textsuperscript{36} synergistic antiviral effects were seen when lactoferrin and acyclovir were tested in vitro against several laboratory and clinical strains of HSV-1 and HSV-2. Our laboratory group has reported\textsuperscript{37} that lactoferrin also reduces the in vitro replication of FHV-1. Therefore, assessment of a potential synergistic effect against FHV-1 for a combination of lactoferrin and the antiviral drugs studied here is warranted.

\textsuperscript{25}Ganciclovir, Hoffmann-La Roche Inc, Nutley, NJ.

References


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