In vitro effects of the active metabolite of leflunomide, A77 1726, on feline herpesvirus-1

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Objective—To determine whether the active metabolite of leflunomide, A77 1726 (A77), inhibits replication of feline herpesvirus-1 (FHV-1) in cell culture.

Study Population—Crandell Rees feline kidney (CRFK) cell cultures.

Procedures—Cell cultures were inoculated with FHV-1 and treated simultaneously with concentrations of A77 ranging from 0 to 200µM. The antiviral effect of A77 was determined by use of conventional plaque reduction assays. The effect of A77 on viral load was determined via real-time PCR analysis, and transmission electron microscopy was used to evaluate the effect of A77 on viral morphology. To determine whether the antiviral effect was attributable to alterations in CRFK cell viability and number, CRFK cells were treated with various concentrations of A77 and stained with Annexin V and propidium iodide to assess apoptosis and a mitochondrial function assay was used to determine cell viability.

Results—Concentrations of A77 ≥ 20µM were associated with substantial reduction in plaque number and viral load. Concentrations ≥ 100µM were associated with complete suppression of plaque formation. At low concentrations of A77, clusters of intracytoplasmic virus particles that appeared to lack tegument and an external membrane were detected. Treatment of uninfected CRFK cell monolayers with A77 was associated with reduction in mitochondrial function with minimal evidence of apoptosis.

Conclusions and Clinical Relevance—Leflunomide may be an alternative to current calcineurin-based immunosuppressive protocols used in feline organ transplantation because of its antiviralpervisual activity. (J Am Vet Med Assoc 2007;231:1010–1015)

Infection secondary to pharmacologic immunosuppression is an important cause of morbidity and death in feline renal transplant recipients. The prevalence of infection in 169 feline renal allograft recipients was 25%.1 Viral infections were thought to comprise 13% of the infections, and 11 of 13 cats with suspected viral infections had severe upper respiratory tract disease, commonly within a month of transplantation.

Feline renal transplant recipients are particularly susceptible to reactivation of latent FHV-1 infection as a result of the stress of illness, surgery, and pharmacologic immunosuppression.2 Exclusion of cats with a history of upper respiratory tract disease as candidates for transplantation is not possible because of the high prevalence of FHV-1 infection in the general population. Also, some cats with latent infection may lack a history of upper respiratory tract disease. Presently, there is no uniformly effective systemic treatment for disease caused by FHV-1.2

The immunosuppressive agent leflunomide [N-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486] is a synthetic organic isoxazole that is metabolized by the intestinal mucosa to its active form, A77. This metabolite inhibits fibroblast, smooth muscle, and T and B lymphocyte proliferation by inhibiting dihydroorotate dehydrogenase, an enzyme in-

ABBREVIATIONS

<table>
<thead>
<tr>
<th>ABBR</th>
<th>DESCRIPTION</th>
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<tr>
<td>FHV-1</td>
<td>Feline herpesvirus-1</td>
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<tr>
<td>A77</td>
<td>A77 1726</td>
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<tr>
<td>HSV-1</td>
<td>Herpes simplex virus-1</td>
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<tr>
<td>CMV</td>
<td>Human cytomegalovirus</td>
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<tr>
<td>CRFK</td>
<td>Crandell Rees feline kidney</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>PFU</td>
<td>Plaque-forming unit</td>
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<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium</td>
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<td>TMFA</td>
<td>Trimethylfluoroanaline</td>
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Leflunomide, an immunosuppressive drug with a relatively low adverse effect profile, is presently approved for treatment of rheumatoid arthritis in humans. Leflunomide controls allograft rejection in small and large animal models and has been used successfully to manage refractory immune-mediated disease in dogs. A recent clinical study revealed that leflunomide possesses substantial immune suppressive potency in human renal and liver transplant recipients and may be safely administered for > 300 days.

In addition to its immunosuppressive effects, leflunomide appears to have antiviral properties, both in vitro and in vivo. The replication of HSV-1, an alphaherpesvirus, and CMV, a betaherpesvirus, is inhibited following treatment of cell monolayers with A77, and leflunomide reduces viral load by 4 to 6 orders of magnitude in animals infected with rat-origin CMV. Results of electron microscopic studies suggest a unique mechanism of action that relates to interference of herpesviral nucleocapsid tegmentation. Leflunomide has been used successfully to treat primary CMV infection and ganciclovir-resistant CMV infection in human transplant patients. Given that HSV-1 is more closely related to FHV-1 than to CMV, we hypothesized that A77 would similarly inhibit the replication of FHV-1. The purpose of the study reported here was to examine the effect of A77 on FHV-1 replication in vitro.

Materials and Methods

Plaque reduction assays—Crandell Rees feline kidney cells were grown in 12-well tissue-culture plates until confluent in minimum essential media with non-essential amino acids and 10% FBS, 7.5mM HEPES, 0.06M NaHCO₃, and 50 µg of ampicillin/mL. The medium was removed, and monolayers were treated with approximately 100 PFUs of FHV-1 suspended in Dulbecco modified Eagle medium plus 10% heat-inactivated FBS, with 0, 5, 10, 20, 35, 50, 100, 150, and 200µM of A77. Virus was permitted to adsorb to 37°C for 1 hour with gentle rocking every 15 minutes. The plaque-purified FHV-1 strain 727 was used for this study. Uninfected, untreated monolayers were also examined. Virus-containing medium was removed, and monolayers were overlayed with 1% sterile carboxymethylcellulose in Dulbecco modified Eagle medium, 1% FBS, 50 µg of ampicillin/mL, and appropriate concentrations of A77. Each concentration was tested in triplicate. All cell cultures were examined daily until visible plaques developed (approx 48 hours). The medium was removed, and cell monolayers were rinsed with PBS solution, fixed with 1% formalin, and stained with crystal violet. Plaques in each well were counted manually via microscopy.

Electron microscopy—For electron microscopy, CRFK cell monolayers were grown on chamber slides, infected with 1,000 PFUs of FHV-1, and treated simultaneously with 0, 5, 10, 15, and 100µM of A77. Monolayers were examined via transmission electron microscopy 20 hours after inoculation after washing with PBS solution. Cells were prepared with Karnovsky fixative in 0.08M PO₄ and pelleted via centrifugation. Pelleted cells were stabilized via addition of liquefied 2% agarose and subsequent cooling to 21°C. Agarose blocks were then fixed on ice for 1 hour in a 0.1M PBS solution containing 1% osmium tetroxide and washed 3 times in cold distilled water. Specimens were fixed on ice in a 0.1% tannic acid solution for 30 minutes, washed 3 times with cold distilled water, and treated with a 2% uranyl acetate solution for 1 hour before dehydration in acetone and embedding overnight in araldite resin. Thin sections were cut with a diamond knife on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined with an electron microscope.

Determination of viral load—The effect of A77 on viral load was determined after incubating CRFK cell monolayers with 0, 5, 10, 20, 50, 100, 150, and 200µM of A77 1 hour prior to inoculation with 100 PFUs of FHV-1. Viral load was determined via quantitative real-time PCR assay in cell monolayers at 36 and 48 hours after inoculation with FHV-1.

Genomic DNA was extracted from approximately 10⁵ CRFK cells by use of a commercially available kit and an automated nucleic acid workstation according to the manufacturer's instructions. The DNA was eluted in 120 µL of nuclease-free water and stored at −20°C prior to PCR assay.

A quantitative PCR system designed to detect the glycoprotein B gene was used to detect FHV-1 DNA. Real-time PCR was performed by use of a real-time PCR instrument and a commercially available mastermix containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl₂, 2.5mM dNTPs, 0.25 U of uracile N-glycosylase (UNG), and 0.625 U of DNA polymerase/ reaction; 400nM of each primer; 80nM of probe; and 5 µL of template. The probe was labeled at the 5′ end with the reporter dye 6-FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). A PCR system for the single-copy chemokine (C-C motif) receptor 3 gene was used as an internal control to test for the integrity of extracted genomic DNA and a standard for quantitation of FHV-1 DNA, as reported. The real-time PCR system targeting this gene was used to quantify genome equivalents and therefore nucleated cell numbers. In parallel, the FHV-1 raw cycle threshold data were extrapolated to a standard curve generated with a cloned FHV-1 PCR fragment to obtain the FHV-1 load. The FHV-1 load and the cell number were combined into a unit FHV-1 load per million nucleated cells, as described.

Assessment of apoptosis and cell viability in uninfected monolayers—Apoptosis was evaluated in uninfected monolayers 36 and 48 hours after treatment with A77 by use of a commercially available Annexin V and propidium iodide staining kit, according to the manufacturer's instructions. All samples were analyzed via flow cytometry within 1 hour of staining. The FL1 channel was used to detect cells stained with Annexin V-FITC, and the FL2 channel was used to detect cells stained with propidium iodide. The positive control sample consisted of CRFK cells treated with buffered
3% formaldehyde (to induce apoptosis) for 30 (36-hour control) or 60 (48-hour control) minutes. Cells stained with both Annexin V-FITC and propidium iodide were considered to be necrotic or late-apoptotic cells, as described.26,27

Cell viability in uninfected monolayers was assessed 96 hours after treatment with 0, 10, 20, 50, 100, 150, 200, and 300µM of A77 by use of an MTS assay. This assay relies on the ability of mitochondria to convert the tetrazolium salt MTS into a water-soluble, colored formazan product, which can be measured spectrophotometrically.27 The CRFK cells and various concentrations of A77 were incubated in 96-well tissue culture plates with or without the MTS dye solution (20 µL/well) for 3 hours prior to determining absorbance at 490 nm. The absorbance was determined 4 times for each concentration of A77 tested. The absorbance value for the A77 alone was subtracted from that of the cells treated with both A77 and the MTS solution. The resultant value was expressed as a percentage of the absorbance value for cells treated with MTS alone. This value is directly proportional to the number of viable cells.

Statistical analysis—The relationships between various concentrations of A77 and plaque numbers, viral load, apoptosis, and the results of the MTS assay were evaluated by use of the Spearman rank correlation test. A 1-sample Student t test was used to evaluate the difference between the results of the apoptosis assay in treated and untreated cells and the result in the positive control wells. Statistical analyses were performed with a standard statistical software package.27 For all comparisons, P < 0.05 was considered significant.

Results

Treatment of CRFK cell monolayers with A77 at the time of inoculation with FHV-1 was associated with a significant (P < 0.001) reduction in plaque formation (Figure 1). No visible plaques were detected with concentrations of A77 ≥ 100µM, and a concentration of 35µM was associated with approximately 50% reduction in plaque numbers, compared with untreated control monolayers.

A marked reduction in viral load as determined via PCR assay was also detected with concentrations of A77 > 20µM, although at lower concentrations, virus load appeared to increase (Figure 2). The increase at lower concentrations was not significant. At 36 hours after inoculation, a concentration of 20µM reduced viral load by 83%, and concentrations ≥ 100µM were associated with reductions of viral load by > 95% (P = 0.005). At 48 hours after inoculation, a concentration of 20µM reduced viral load by 18% and concentrations ≥ 100µM were associated with reductions of viral load by > 98% (P = 0.01).

Forty-two fields of untreated, infected cells were examined at low power via transmission electron microscopy (approx 700 cells). From these, 29 fields (approx 60 cells) were selected for examination at high magnification because they appeared to contain heavily infected cells. Normal maturation of FHV-1 was observed in these untreated cell cultures, resulting in the accumulation of large numbers of intact virus particles within the cytoplasm of infected cells. A total of 99 fields (26, 48, and 25 at 5, 10, and 15 µM of A77, respectively) of treated, infected cells were examined at low power, and from these, 24 fields (approx 50 cells) were selected for examination at high magnification. Although intact virus particles were frequently identi-
1013

fied within the cytoplasm and extracellular space, 10 of the 24 fields contained either clusters of intracytoplas-
mic virus particles lacking both tegument and an enve-
lope or isolated naked nucleocapsids in the cytoplasm (Figure 3). The total number of naked nucleocapsids found in treated cells by use of this method was 102 (61, 24, and 17 at 5, 10, and 15µM A77, respectively). Such structures were not observed in any of the fields of untreated cells examined (P < 0.001). No virus particles were seen in monolayers treated with 100µM of A77.

Treatment of uninfected CRFK cell monolayers with A77 was not associated with apoptosis or cell death at most concentrations of A77 tested (Figure 4), both at 36 (P = 0.15) and 48 hours (P = 0.88), although at 48 hours at an A77 concentration of 200µM only, the proportion of living cells approximated that for cells treated with formaldehyde. The MTS assay revealed a progressive reduction in mitochondrial function with increasing concentrations of A77 (P < 0.001; Figure 5). Concentrations of A77 ≥ 100µM were associated with a slight alteration in the light microscopic appearance of the CRFK cell monolayer, consisting of rounding up of rare cells.

Discussion

In this study, A77 inhibited viral replication and plaque formation in vitro. The mechanism of antiviral activity may be interference with assembly of virions. The effect of A77 on FHV-1 morphology in feline cell cultures was more marked than that reported on HSV-1 morphology in human cell line cultures. Although the A77-treated virus particles in human cell cultures lacked tegument or had marked alterations in the tegument, they still had an external capsule or envelope. In the present study, some of the A77-treated intracytoplasmic virus particles lacked both tegument and an enve-

Figure 3—Transmission electron micrographic views of FHV-1–infected CRFK cell cultures incubated for 20 hours with low concentrations of A77. A—Ten micromole A77. Notice intact virus particles (thin arrows) and viral particles lacking tegument and envelope (thick arrows). Bar = 0.2 µm. B—Fifteen micromole A77. Notice cluster of intracytoplasmic viral particles lacking tegument and envelope. Bar = 0.2 µm.

Figure 4—Mean ± SD percentages of living CRFK cells cultured without A77 and with various concentrations of A77 for 36 and 48 hours. The bars on the far right represent the formaldehyde control samples.

Figure 5—Mitochondrial cleavage of MTS in CRFK cells at various concentrations of A77, expressed as a percentage of the absorbance value of cells treated with MTS alone (control sample).
velope. No such virus particles were detected in untreated cells. The initial increase in viral DNA load at low concentrations of A77 detected via real-time PCR assay occurred despite reduction in plaque formation. This may reflect an accumulation of unenveloped virus particles. At higher concentrations, the large decline in viral DNA load suggested that the viral replication cycle was blocked at some point at or before DNA replication. The relationship between these different effects requires further elucidation.

These findings have major clinical implications in 2 areas. First, FHV-1 infection is not just a problem in feline renal transplant patients but is widely prevalent in the cat population as a whole. The illness and economic loss caused by FHV-1 infection in commercial and private catteries and humane shelters are substantial. There are presently several treatment options available for cats infected with FHV-1; however, efficacy of the options seems to vary among individuals, and some cats respond poorly to any treatment. Leflunomide has at least 2 mechanisms of action. If the antiviral component can be identified and isolated from the drug's general immunosuppressive effects, it may be considered for use as a treatment or prophylaxis for FHV-1 infections in individuals unresponsive to conventional treatments and in susceptible populations of cats.

Calcineurin-based immunosuppression has been the mainstay of both human and companion animal clinical transplant programs for years. Cyclosporine is the primary immunosuppressive agent presently used to maintain kidney allografts in dogs and cats. Although relatively safe and highly effective, there are substantial and potentially lethal adverse effects associated with administration of calcineurin inhibitors. Diabetes mellitus, nephrotoxicity, and an increased incidence of neoplasia, particularly lymphoma, have been associated with administration of cyclosporine in human and feline renal transplant recipients. In addition, although cyclosporine is effective in preventing acute renal allograft rejection, it has no effect on graft loss caused by chronic rejection. For these reasons, there has been a movement away from calcineurin-based immunosuppressive protocols.

Leflunomide has many characteristics that make it an attractive alternative to cyclosporine for use in transplantation. Leflunomide has also been used to successfully treat corticosteroid-resistant autoimmune hemolytic anemia and systemic histiocytosis in dogs. Inhibition of B and T lymphocytes results in a reduction in T-cell–mediated graft destruction and prevention of alloantibody production. Leflunomide also inhibits growth factor–stimulated (platelet-derived growth factor, fibroblast growth factor, transforming growth factor) proliferation of smooth muscle cells and fibroblasts. Both cell types appear to play a pivotal role in the development of chronic allograft rejection and organ failure. Finally, the antiviral effects of leflunomide could reduce the prevalence of upper respiratory tract infections in feline renal transplant recipients. At human dosages, leflunomide causes gastrointestinal toxicity in dogs because of accumulation of a metabolite, TMFA. Fortunately, canine lymphocytes are far more susceptible than human lymphocytes to the effects of the active agent, A77. In dogs, leflunomide effectively achieves immunosuppression at much lower orally administered doses. Although complete pharmacokinetic and toxicologic studies need to be completed, early data collected from our laboratory cats suggest that TMFA is not as toxic to cats as it is to dogs. Cats metabolize the drug at a slower rate and require approximately half the orally administered dose required in dogs to achieve effective blood concentrations. Both cats and dogs with diminished renal function may be subject to TMFA toxicosis because of excretion by the kidneys.

Some authors are concerned that, in its present formulation, the immunosuppressive properties of leflunomide will likely reduce its potential as an antiviral treatment for transplant recipients already receiving other immunosuppressive treatment. The risk of further immunosuppression by this agent may represent a strong contraindication in this population. However, the immunosuppressive effects of the drug may allow it to replace other agents presently used; therefore, it may not be just an adjunctive agent used for its antiviral effect. Leflunomide has effectively controlled CMV infections in human renal transplant patients and a bone marrow transplant patient without evidence of toxicosis or increased risk of infection.

Further studies are needed to determine the long-term effects of leflunomide on prevention of acute and chronic allograft rejection and the prevalence of immunosuppression-related viral infections in humans and other animals.

The Annexin V and propidium iodide assays revealed that A77 had a cytotoxic effect on the feline kidney cells only at a concentration of 200 µM after 48 hours of treatment. However, there was a dose-dependent reduction of mitochondrial function as determined by the MTS assay and light microscopic changes to the monolayer at concentrations ≥ 100 µM. The ability of leflunomide to inhibit protein kinases might also explain the change in mitochondrial function detected in the present study. Multiple protein tyrosine kinases have been detected in mitochondria, and it is suggested that tyrosine kinases are involved in the regulation of oxidative phosphorylation. Nonreceptor src kinases represent the first tyrosine kinases determined to reside in mitochondria. Src-family kinases have also been implicated as critical regulators of a large number of intracellular signaling pathways, most notably those controlling cell growth and adhesion. Inhibition of such kinases might also help to explain the light microscopic changes seen in the CRFK cells. Substantial ultrastructural differences between untreated and treated cells were not observed in this study.
References