Use of interfering RNAs targeted against feline herpesvirus 1 glycoprotein D for inhibition of feline herpesvirus 1 infection of feline kidney cells

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Objective—To evaluate the use of RNA interference targeted against feline herpesvirus 1 (FHV-1) glycoprotein D for inhibition of FHV-1 infection of feline kidney cells.

Sample Population—Crandell-Rees feline kidney cells.

Procedures—Crandell-Rees feline kidney cells were transfected with small interfering RNAs (siRNAs) that were designed to inhibit expression of FHV-1 glycoprotein D. The effectiveness of the treatment was determined via measurement of amounts of glycoprotein D mRNA, intracellular glycoprotein D, and glycoprotein D expressed on the surface of infected cells and comparison with appropriate control sample data.

Results—2 of 6 siRNAs tested were highly effective in reducing expression (ie, knockdown) of glycoprotein D mRNA; there were 77% and 85% reductions in mRNA in treated samples, compared with findings in the control samples. The knockdown of glycoprotein D mRNA resulted in reduced glycoprotein D protein production, as evidenced by 27% and 43% decreases in expression of glycoprotein D on the surface of siRNA-treated, FHV-1-infected cells and decreased expression of the protein within infected cells, compared with control samples. Treatment with these siRNAs also resulted in inhibition of FHV-1 replication, with reductions of 84% and 77% in amounts of virus released into cell culture supernatant, compared with findings in control samples.

Conclusions and Clinical Relevance—2 chemically produced siRNAs that targeted the glycoprotein D gene significantly reduced FHV-1 titers in treated cells, suggesting that glycoprotein D is necessary for production of infective virions. This gene is a potential target for RNA interference as a means of inhibition of FHV-1 infection of feline cells. (Am J Vet Res 2009;70:1018–1025)

Feline herpesvirus 1 is a linear double-stranded virus that causes approximately 50% of upper respiratory tract infections and induces the most severe respiratory tract disease in domestic cats. This virus is labile in nature but maintains itself via latent infections in its host. Killed and modified-live FHV-1 vaccines are available, but because the virus is poorly immunogenic, the vaccines do not prevent infection and induce only partial protection from clinical disease. Acute infections are usually localized to the respiratory tract, and clinical signs, although potentially severe, typically resolve in a few weeks. However, chronic disease in latently infected cats is problematic. Repeated recrudescence, mainly affecting the eye, can potentially lead to blindness. Antiviral medications approved for treatment of a related virus, herpes simplex virus type 1, in humans are only minimally effective for treatment of cats with chronic FHV-1 infection. Therefore, development of a new treatment for FHV-1 infection in cats would be beneficial.

Feline herpesvirus 1 glycoprotein D (an envelope protein) is the viral hemagglutinin and an inducer of virus-neutralizing antibodies. The glycoprotein D gene also appears to be highly conserved. Feline herpesvirus 1 glycoprotein D may play an important role in the restriction of the host range of the virus to feline cells. It is unknown whether FHV-1 glycoprotein D is a viral attachment protein; in the related virus, herpes simplex virus type 1, glycoprotein D is an attachment protein and is essential for entry of the virus into cells.
Small interfering RNAs can be produced chemically and delivered to cells to silence specific genes of interest (by inhibiting expression of those genes by causing destruction of mRNA). Chemically produced siRNAs of 21 nucleotides have been used routinely for RNAi experiments since it was discovered that they initiate RNAi in mammalian cells. In mammalian cells, RNAi can also be activated by longer siRNAs (ie, 27-mers). The 27-mers are actually more potent inducers of RNAi than 21-mers; these longer siRNAs are processed by the endoribonuclease Dicer, and the resultant siRNAs are therefore more efficiently incorporated into an RNA-induced silencing complex.

In previous experiments, RNAi (initiated by exogenous siRNAs) has been used successfully to determine the function of viral genes; it has been used therapeutically for mammalian viral infections. One method of reducing viral replication in cells is suppressing production of viral attachment proteins. The purpose of the study reported here was to evaluate the use of RNAi targeted against FHV-1 glycoprotein D as a means of inhibiting FHV-1 infection of feline kidney cells. We hypothesized that FHV-1 glycoprotein D, like its homolog herpes simplex virus type 1 glycoprotein D, is necessary for viral infection.

Materials and Methods

**Cells and viruses**—Crandell-Rees feline kidney cells were propagated and maintained in DMEM with supplemental 5% heat-inactivated fetal bovine serum and standard concentrations of penicillin, streptomycin, and amphotericin B at 37°C with 5% CO₂, in an incubator. The FHV-1 strain used was the prototype strain C-27. A calicivirus wild-type strain was used as a control in the study.

**siRNAs and transfection**—Both 21-mer and 27-mer siRNAs were designed to target the glycoprotein D gene of FHV-1 (Appendix 1). These sequences were compared with known sequences in the GenBank database to decrease off-target effects by avoiding similar sequences in the feline genome. The portion of the FHV-1 glycoprotein D coding sequence that was targeted by the siRNAs was identical in each of the isolates in the GenBank database used in this study, and in an additional 10 isolates (collected in 2003 through 2006) that were obtained from the Clinical Virology Laboratory, College of Veterinary Medicine, University of Tennessee. Negative control 21-mer and 27-mer siRNAs were obtained from commercial sources. A cyanine-5-labeled control siRNA was used as a transfection control agent. Transfections were performed with a transfection reagent in a modified Eagle minimal essential medium, according to the manufacturer's protocol. Five microliters of the transfection reagent was used to transfect 100 µM of 21-mer siRNAs well and 50 nM of 27-mer siRNAs/well in 6-well plates. Approximately 5 x 10⁴ CRFK cells diluted in DMEM with supplemental 10% fetal bovine serum were added to each well containing the transfection mixtures. Plates were incubated for 24 hours prior to infection with FHV-1. Each siRNA was tested in duplicate, and functional siRNAs were retested for a total of 3 experiments/siRNA. Additional control conditions for each experiment included uninfected, nontransfected CRFK cells; infected, nontransfected CRFK cells; and nontransfected CRFK cells infected with feline calicivirus. The latter was used as an interferon β control condition because feline calicivirus is an RNA virus that activates interferon β in CRFK cells. The nontransfected CRFK cells were used as a control to determine toxic effects resulting from transfection of the cells.

**Plaque assays**—The FHV-1 strain C-27 was grown in CRFK cells until a 50% cytopathic effect was achieved. The titer of the virus was determined via plaque assay as previously described, except that an agarose overlay was not used. Instead, serum from a cat with an immunofluorescent antibody titer of > 2,560 was used at a dilution of 1:50.

Transfected CRFK cells were infected with FHV-1 at an MOI of 0.1. One hour after incubation, the cells were washed with DMEM, and fresh DMEM with supplemental 10% fetal bovine serum was added to each well. Infected cells were incubated for 48 hours, after which 500 µL of cell culture medium was removed from each well and stored at ~80°C for plaque assay. Infective virus titers (determined as PFUs) were determined for each well via plaque assay.

**Western blot analysis**—Proteins from FHV-1-infected cells were treated with SDS sample buffer; electrophoresis was performed on a 10% polyacrylamide gel, and gel products were transferred to a nitrocellulose membrane. The membrane was blocked overnight and probed with FHV 7-5 monoclonal antibody for 1 hour on a shaker at room temperature (approx 22°C). The membrane was washed 5 times with PBS solution containing 0.05% Tween 20 and probed with peroxidase-labeled goat anti-mouse IgG for 1 hour on a shaker at room temperature. Five additional washes were performed, and the protein was detected by enhanced chemiluminescence. A biotinylated protein ladder was used to estimate protein size.

**Hemagglutination inhibition assay**—The assay was performed as previously described. Two-fold serial dilutions of monoclonal FHV 7-5 were tested. The FHV-1-infected CRFK cells were treated with Tween 80 and ethylether as previously described and used as the antigen for the assay (4 hemagglutination units/25 µL); 0.3% (vol/vol) feline RBCs were added to each well.

**Flow cytometry**—Prior to infection with FHV-1 (ie, 24 hours after transfection), the transfection control well was evaluated to determine transfection efficiency. The cells were treated with trypsin, washed in PBS solution and flow buffer (60 mL of 0.5% sodium azide solution, 87 mL of PBS solution, and 3 mL of fetal bovine serum), and resuspended in 1 mL of PBS solution. Transfection efficiency was determined via analysis of intracellular cyanine-5 fluorescence by use of a flow cytometer. Following FHV-1 infection and a 48-hour incubation period, the cells in each test and control well were treated with trypsin, washed with PBS solution, and resuspended in 1 mL of PBS solution/sample. One hundred microliters of each suspension was removed...
and placed on ice for RNA extraction, and the remainder of each sample was processed for flow cytometry. The flow cytometry samples were washed in flow buffer, and the cells were pelleted. Each sample was stained with the primary monoclonal antibody FHV 7-5 at a dilution of 1:50 in PBS solution for 1 hour on ice. The cells were then washed and treated with 2 µL of the secondary antibody fluorescein isothiocyanate-labeled F(ab'), rabbit anti-mouse IgG' for 1 hour on ice. After a final wash, the cells were resuspended in 1 mL of PBS solution/sample and analyzed via flow cytometry.

RNA extraction and real-time RT-PCR assay—Total RNA was extracted from 100-µL aliquots of each test and control sample according to the kit manufacturer's protocol. The RNA samples were treated with 2 on-column DNase digestions and diluted 1:1,000 to reduce DNA contamination. Purified RNA samples were stored at –80°C until tested by use of a real-time RT-PCR assay. Primers and probes for the real-time RT-PCR assay were developed by use of a computer program to detect FHV-1 glycoprotein D and interferon β mRNA (Appendix 2). To standardize RNA concentration, 28S rRNA was used as a control for each sample. The real-time RT-PCR assay was performed for each transcript by use of a 1-step quantitative real-time RT-PCR kit in a thermal cycler. Five microliters of diluted RNA was used in 23-µL (total volume) reactions, which contained 200nM of each probe and 300nM of each primer. The reaction conditions for glycoprotein D and interferon β involved RT at 42°C for 30 minutes and an initial heat step at 95°C for 2 minutes to activate the hot-start Taq polymerase, followed by 45 cycles at 95°C for 15 seconds, at 50°C (glycoprotein D) or 60°C (interferon β) for 60 seconds, and at 72°C for 30 seconds. The reaction conditions for 28S rRNA involved RT at 50°C for 30 minutes and at 95°C for 2 minutes, followed by 45 cycles at 95°C for 15 seconds, at 62°C for 60 seconds, and at 72°C for 30 seconds. To rule out excessive DNA contamination, RTase negative control samples were run with platinum Taq instead of the reverse transcriptase in the kit. Each set of samples was run with mRNA standard dilutions to validate mRNA quantitation.

mRNA standards and generation of standard curves—The mRNA standards for glycoprotein D and interferon β were produced by cloning the PCR products into plasmid vectors and transducing Escherichia coli. Recombinant plasmids were isolated, sequenced, linearized, and used for in vitro transcription. The mRNA transcripts were treated with DNase and purified. These standards were used to create standard curves for quantitation of the glycoprotein D and interferon β mRNA transcripts that were isolated from the samples. The numbers of RNA copies were estimated based on the molecular weights of the RNA standards and the RNA concentrations. Ten-fold serial dilutions were prepared, and aliquots of each dilution were stored at –80°C and used only once.

Standard curves were generated from data obtained via testing of dilutions of the standard RNAs by use of a real-time RT-PCR procedure and thermal cycler software. The intra- and interassay coefficients of variation of the reactions were determined by use of dilutions of the standard RNAs as previously described. To ensure that the standard RNAs and the target RNAs were amplified with similar efficiencies (based on a calculation as follows: Efficiency = [10^{-1/slope} – 1]) four 10-fold serial dilutions of RNA extracted from an FHV-infected control well and RNA extracted from a calcivirus-infected interferon β control well were also prepared and evaluated via the real-time RT-PCR procedure.

Statistical analysis—The plaque assay results for the glycoprotein D–specific siRNA tests and data from the control samples were compared by use of a univariate ANOVA because all assumptions were met based on the Levene test for equality of variances and the Shapiro-Wilk test for normality. Values of P < 0.05 were considered significant.

Results

Effects of FHV-1 glycoprotein D–specific siRNA on glycoprotein D mRNA and protein expressions—To examine whether glycoprotein D expression could be reduced (or knocked down) by RNAi, three 27-mer and three 21-mer siRNAs were designed to target 5 areas of glycoprotein D mRNA. The siRNAs were transfected into cells prior to infection with FHV-1. The CRFK cells were effectively transfected with the transfection reagent (efficiency, ≥ 95% based on results from a transfection control siRNA [data not shown]). Toxic effects on cells were evaluated via microscopic examination. Signs of toxic effects were minimal, with only mild granularity observed in cells transfected with 21-mer siRNAs. Transfection with 100nM concentrations of 27-mers resulted in some cell death and release of cells from the well; however, the toxic effects were reduced to development of mild granularity with use of 50nM concentrations.

A quantitative real-time RT-PCR procedure was used to determine the reduction in glycoprotein D mRNA expression (ie, knockdown) 48 hours after infection. For each sample, mRNA copy numbers were estimated from a standard curve generated from dilutions of standard glycoprotein D RNA. The standard curve spanned 5 orders of magnitude and had linearity over the entire range used for quantitation of mRNA. The results were reproducible,
with an intra-assay coefficient of variation (based on copy numbers) of 10% to 21% (0.5% to 1.24% based on Ct values) and an interassay coefficient of variation (based on copy numbers) of 34% to 52% (1.33% to 3.40% based on Ct values). The theoretical limit of detection was 20 copies of glycoprotein D mRNA.

Of the siRNAs tested, one of the 27-mers (gD1) and one of the 21-mers (G3) were highly effective in achieving knockdown of glycoprotein D mRNA. For gD1, mean ± SD number of copies of glycoprotein D mRNA/5 μL of RNA was 14,147 ± 3,687, compared with the findings in control samples transfected with the negative control siRNA (61,328 ± 14,962 copies of glycoprotein D mRNA/5 μL of RNA). For G3, the mean number of copies of glycoprotein D mRNA/5 μL of RNA was 7,009 ± 1,245, compared with the findings in control samples transfected with the negative control siRNA (46,894 ± 17,046 copies of glycoprotein D mRNA/5 μL of RNA). These reductions represented knockdowns of 77 ± 7% and 85 ± 7%, respectively. The remainder of the siRNAs had minimal to moderate effects, compared with findings in control samples transfected with negative control siRNAs. The least functional siRNAs (responsible for approx 30% knockdown each) were the 27-mer gD2 and the 21-mer G1, both of which targeted the same region of the coding sequence. Therefore, gD1 and G3 were chosen for additional experiments.

To determine whether these siRNAs were also functional with an increased infectious dose of virus, gD1 and G3 siRNAs were evaluated in cells that were infected with FHV-1 (MOI = 1) 24 hours after transfection and tested for mRNA knockdown 24 hours after infection. Under these reaction conditions, G3 was effective; however, the 27-mer gD1 was not effective (data not shown).

Glycoprotein D protein synthesis was assessed via flow cytometry in glycoprotein D–specific siRNA-treated cells and compared with findings in negative control siRNA-treated cells. For this test, FHV-1 monoclonal antibodies were characterized via western blot analysis, flow cytometry, virus neutralization, and hemagglutination inhibition assays. One monoclonal antibody (FHV 7-5) reacted with an antigen that was approximately 50 to 60 kDa (Figure 1); this antigen was detected on the surface of infected cells via flow cytometry and inhibited hemagglutination of feline cells (Figure 2). These results suggested that this monoclonal antibody detected FHV-1 glycoprotein D.10 However, FHV 7-5 did not neutralize virus infectivity in the absence of complement as expected, but this is a variable characteristic based on epitope.9 Therefore, FHV 7-5 was used to assess the amount of glycoprotein D present on the surface of FHV-1–infected cells that were transfected with negative control siRNAs or with glycoprotein D–specific siRNAs.

**Figure 1**—Western blot analysis with an arbitrary antibody (FHV 7-5) reacted with an antigen that was approximately 50 to 60 kDa. A—Uninfected CRFK cells. B—Uninfected CRFK cells treated with negative control siRNA. C—Feline herpesvirus 1–infected cells. D—Feline herpesvirus 1–infected cells treated with negative control siRNA. E—Negative control siRNA–treated, FHV-1–infected cells. F—Negative control siRNA–treated, FHV-1–infected cells in a representative gD1 experiment. 

**Figure 2**—Results of hemagglutination inhibition testing performed with monoclonal FHV 7-5 and Tween 80-ethyl ether–treated FHV–infected CRFK cells (with 0.5% [vol/vol] feline RBCs as previously described). In the photograph, 7 wells are viewed from above. Each number represents the inverse of 2-fold serial dilutions of antibody within the well; C represents the control well, which lacked FHV-1.

**Figure 3**—Results of flow cytometric analysis (events/channel vs fluorescence intensity [logarithm base 10]) to determine the effect of FHV-1 glycoprotein D–specific siRNAs on expression of glycoprotein D protein on the surface of FHV–infected CRFK cells. Cells were transfected with G3, gD1, or negative control siRNA 24 hours prior to FHV-1 infection (MOI = 0.1). Forty-eight hours following infection, cells were incubated with FHV 7-5 monoclonal antibody (a glycoprotein D–specific monoclonal) and fluorescein isothiocyanate–labeled F(ab)2, rabbit anti-mouse IgG. A—Uninfected CRFK cells. B—Uninfected CRFK cells in a representative G3 experiment. C—Feline herpesvirus 1–infected cells treated with G3. D—Feline herpesvirus 1–infected cells treated with gD1. E—Negative control siRNA–treated, FHV-1–infected cells in a representative G3 experiment. F—Negative control siRNA–treated, FHV-1–infected cells in a representative gD1 experiment. Fluorescence intensity increases from left to right; for each sample, 104 cells were analyzed. Each experiment was repeated 3 times with similar results.
The gD1 and G3 siRNAs decreased glycoprotein D protein expression by 27 ± 3% and 43 ± 8%, respectively, compared with the negative siRNA-transfected control findings (Figure 3).

Effect of FHV-1 glycoprotein D–specific siRNAs on virus replication—To determine whether knockdown of glycoprotein D mRNA affects virus replication, plaque assays were performed to quantify the amount of infective virus released into cell culture supernatants. In cells transfected with gD1, mean ± SD virus titer was 830,000 ± 378,994 PFUs/mL, compared with the findings in samples transfected with the negative control siRNA (5,170,000 ± 1,258,306 PFUs/mL). In cells transfected with G3, the mean virus titer was 1,190,000 ± 1,069,720 PFUs/mL, compared with the findings in samples transfected with the negative control siRNA (5,167,000 ± 288,675 PFUs/mL). Viral replication was significantly inhibited by 84 ± 6% with gD1, compared with findings in negative control siRNA-transfected FHV-1–infected cells (P = 0.003), and by 77 ± 19% with G3, compared with findings in nontransfected FHV-1–infected cells (P = 0.003).

Assessment of the specificity of glycoprotein D–specific siRNAs—To confirm virus replication suppression via knockdown of glycoprotein D mRNA, it was necessary to distinguish that effect from non–sequence-specific destruction of all viral mRNA transcripts as a result of induction of the type 1 interferon pathway.25 In previous studies,26,27 interferon induction in epithelial cells has been ruled out by use of interferon β detection methods. A quantitative real-time RT-PCR assay for detection of interferon β mRNA in feline cells was designed and used to estimate copy numbers on the basis of a standard curve derived from analysis on an RNA standard. The standard curve generated by dilutions of the interferon β standard RNA spanned 6 orders of magnitude and had linearity over the entire range used for quantitation of mRNA. The results were reproducible, with an intra-assay coefficient of variation (based on copy numbers) of 7% to 43% (0.38% to 2.88% based on Ct values) and an interassay coefficient of variation (based on copy numbers) of 16% to 41% (0.67% to 2.87% based on Ct values). The theoretical limit of detection was 25 copies of interferon β mRNA. Results indicated that the interferon β detected in siRNA-treated cells was negligible (approx 33 copies), compared with findings in the control sample (calcivirus-infected cells [approx 20,000 copies]), and was similar to the background level detected in infected cells that were not transfected with siRNAs (approx 43 copies). Therefore, the suppression of viral replication was the result of the knockdown of glycoprotein D mRNA and not a result of type 1 interferon production.

Discussion

In previous experiments,18,19,31 RNAi has been manipulated for the determination of viral gene function, and results of the present study have indicated that RNAi can be effectively used to knockdown FHV-1 glycoprotein D mRNA in CRFK cells when those cells are transfected prior to infection with the virus. Knockdown of FHV-1 glycoprotein D mRNA resulted in a decrease in the amount of glycoprotein D detected on the surface of infected cells, independent of the production of type 1 interferon. Because of the decrease in the amount of that protein on the viral envelope, the amount of infective virus was reduced, compared with findings in control samples. Feline herpesvirus 1 glycoprotein D, like its counterpart in herpes simplex virus type 1, is essential for the production of infective virions in vitro.

On the basis of the results of our study, we speculate that FHV-1 glycoprotein D is potentially involved with entry of the virus into cells. The FHV-1 glycoprotein D may be involved with viral attachment, similar to the function of its homolog in herpes simplex virus type 113–15 and in equine herpesvirus 1.32 Initiation of herpesvirus infection requires attachment of virions to the host cell, followed by fusion of the virus envelope and cellular cytoplasmic membrane during penetration.24 For herpes simplex virus type 1, glycoprotein D is necessary for secondary attachment of the virus to the host cell and is also required for cell penetration.13–15 Therefore, another possibility for the function of glycoprotein D in FHV infection is viral penetration of the cell.

The mechanisms of entry differ among alphaherpesviruses. Varicella-zoster virus lacks a glycoprotein D homolog, and glycoprotein D in Marek’s disease virus is not essential for infectivity.14,33 Pseudorabies virus glycoprotein D deletion mutants still retain considerable infectivity, and other glycoproteins appear to be essential for penetration of this virus.33,36 Bovine herpesvirus 1 glycoprotein D is essential to viral infection and is also involved in the viral entry process. It has been suggested that bovine herpesvirus 1 glycoprotein D ensures a penetration-competent conformation of other surface glycoproteins rather than directly causing membrane fusion33; therefore, the aforementioned glycoprotein D appears to exert its effects between the processes of virus binding and membrane fusion.37 Another potential function of FHV-1 glycoprotein D is viral egress; however, glycoprotein D is not required for egress of pseudorabies virus,36 and glycoproteins other than glycoprotein D are necessary for fusion between the virion envelope and the outer nuclear membrane in herpes simplex virus.38

Neutralizing antibodies are produced against FHV-1 glycoprotein D.9 This supports the suggestion that FHV-1 glycoprotein D is involved with viral attachment. Attempts to neutralize virus infectivity with the monoclonal antibody used in the current study were performed while characterizing the antibody. The monoclonal antibody did not neutralize virus infectivity, but this is a variable characteristic that is based on epitope.9

Both 21-mer and 27-mer synthetic siRNAs were used in the present study. The 27-mers are more potent inducers of RNAi, compared with 21-mers, and are also potentially functional at sites refractory to RNAi by both types of siRNAs. A computer program designed to predict the secondary structure of
single-stranded nucleic acids was used to predict the glycoprotein D mRNA structure, and we discovered that despite the amenable sequence, the target site is likely self-annealed in a helix in its energetically most favorable structure (data not shown), which has been previously shown to inhibit RNAi.

Also of interest in our study, the 27-mer siRNA (gD1) was not functional in knockdown of glycoprotein D mRNA 24 hours after infection of cells (MOI = 1), but the 21-mer (G3) was functional. We attribute this to the fact that, unlike the 21-mer, the 27-mer has to be processed by the endoribonuclease Dicer. Ribonucleic acid silencing is generally maximal at approximately 24 hours after transfection. However, in an RNAi study in rotavirus, the highest suppression occurred when cells were infected with rotavirus 72 hours after transfection with siRNAs, suggesting that some of the elements of RNAi could be induced or activated by the presence of siRNAs, thereby increasing their effective concentration inside the cell.

In the present study, the 27-mer siRNAs were toxic to cells in a concentration-dependent manner, likely resulting from off-target effects with increased concentrations of siRNA leading to a stress response within the cell. Therefore, the 27-mer siRNA (gD1) was determined to be less toxic than the 21-mer siRNA (G3) and functional at a comparatively lower concentration.

Off-target effects may include stimulation and suppression of expression of genes unrelated to the intended target. These effects appear to be sequence-related; siRNAs may cross-react with mRNAs of limited sequence homogeneity. Therefore, identification of the same phenotype by use of multiple siRNAs targeting the same gene increases the confidence that the knockdown of the intended gene can be attributed to that phenotype. We were able to show the same effects with 2 siRNAs that targeted different areas of the glycoprotein D mRNA, which strengthened our findings.

Feline herpesvirus 1 glycoprotein D is a suitable target for suppressing FHV-1 viral infection in cells. It is therefore a potential target for antiviral treatment, although, as a structural protein, it is not transcribed until late in the course of infection and may not be as good a target as a gene that is transcribed early in the infection process. However, use of siRNAs that target this gene could be used successfully in combination with siRNAs that target additional FHV-1 genes, thereby reducing the potential for development of escape mutants and likely increasing the effectiveness of treatment, compared with that achieved via targeting of a single gene.

References

17. Kim DH, Behlke MA, Rose SD, et al. Synthetic dsRNA Dicer

a. American Type Culture Collection, Manassas, Va.
b. Cambrex, Charles City, Iowa.
c. Atlanta Biologicals, Lawrenceville, Ga.
d. Provided by the Clinical Virology Laboratory, College of Veterinary Medicine, University of Tennessee, Knoxville, Tenn.
e. Ambion-Applied Biosystems, Austin, Tex.
f. BD; Corvallie, Iowa.
g. Lipofectamine 2000, Invitrogen, Carlsbad, Calif.
h. Opti-MEM, Invitrogen, Carlsbad, Calif.
i. Sigma Aldrich, St Louis, Mo.
k. Provided by Dr. Chris Grant, Custom Monoclonals International, Sacramento, Calif.


### Appendix 1

Small interfering RNAs designed to target the glycoprotein D gene of FHV-1.

<table>
<thead>
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<th>siRNA</th>
<th>Antisense sequence (5’ → 3’)</th>
<th>Location (bp range)</th>
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<td>gD1</td>
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<td>gD2</td>
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<td>G3</td>
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*Bold letters represent guide sequence subsequent to processing by Dicer; lowercase letters represent 3’ overhangs; uppercase letters represent guide sequence; lowercase letters represent 3’ overhangs.*

Appendix 2 appears on the next page
### Appendix 2

Primers and probes used for real-time RT-PCR procedures.

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<thead>
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<th>Primer or probe</th>
<th>Sequence (5' → 3')</th>
<th>Location (bp range)</th>
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<tr>
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<tr>
<td>Reverse</td>
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<td>1564–1573</td>
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<tr>
<td>Probe</td>
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<td>1700–1681</td>
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<td><strong>Interferon β</strong></td>
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*Probe designed from sequence obtained from real-time RT-PCR product from CRFK cells.*