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Efficacy of S26308 against Guinea Pig Cytomegalovirus Infection†

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Prophylactic use of antiviral agents against cytomegalovirus (CMV) is particularly indicated for the immunocompromised host because morbidity and mortality due to CMV occur most frequently following immunosuppression. We have evaluated the new Riker compound S26308 for its therapeutic and prophylactic antiviral activity against CMV in guinea pigs. The efficacy of the compound was assessed in vitro in guinea pig embryo cells and in vivo in both immunocompetent and immunocompromised guinea pigs. Guinea pig CMV plaque formation was reduced only in cells treated with S26308 prior to virus infection. The antiviral activity remained even when the compound was removed after virus absorption and was due to neither virus destruction nor inhibition of cell growth. The frequency of viremia was reduced in guinea pigs for which S26308 therapy was initiated 24 h prior to virus inoculation compared with sham-treated animals. This reduction in the frequency of viremia did not prevent virus spread to target tissues but did result in a reduction of the severity of CMV-induced disease in immunocompromised guinea pigs. Low levels of interferon were detected in supernatants of S26308-treated cells, and interferon was detected in the serum of guinea pigs given S26308. These results indicate that S26308 can induce interferon and reduce CMV infectivity in vivo and in vitro when used prophylactically. This antiviral activity, although modest, was accompanied by beneficial effects on CMV-induced morbidity and mortality. Prophylactic use of S26308 in combination with other therapeutic agents may be a useful strategy against CMV infections.

A broad spectrum of morbidity and mortality is associated with infections of humans with cytomegalovirus (CMV). Few antiviral agents have shown efficacy against CMV infections, and there is a clear need for new potent agents that can be used for the prevention and treatment of CMV infections. The antiviral compound 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) is the most potent agent against CMV described to date (5, 7, 16, 18). However, its usefulness remains limited to specific sites of CMV infections, such as the eye and gastrointestinal tract (5, 6, 14, 19). In addition, mutants of human CMV resistant to DHPG have been isolated (2), and development of CMV retinitis in a patient treated with DHPG for CMV colitis has been reported (17). Evaluation of the efficacy of candidate antiviral compounds requires not only testing in vitro, but also in vivo studies in a well-defined experimental model. The guinea pig model of CMV infection is well suited for testing candidate antiviral compounds because the pathogenesis of the viral infection in this animal model closely approximates that in the human host (1).

The new Riker drug S26308, 1-isobutyl-1*H*-imidazo(4,5-*c*)quinolin-4-amine (Fig. 1), has shown efficacy against herpes simplex virus infections in an experimental model (C. J. Harrison, L. Jenski, R. Miller, T. Voychehovski, and D. I. Bernstein, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 383, p. 167, 1986; R. L. Miller, L. M. Imbertson, M. J. Reiter, D. S. Schwartzmiller, S. E. Pecore, and J. F. Gerster, 25th ICAAC, abstr. no. 775, p. 235, 1985). The compound apparently induces significant levels of interferon in guinea pigs (R. L. Miller, L. M. Imbertson, M. J. Reiter, S. E. Pecore, and J. F. Gerster, 26th ICAAC, abstr. no. 385, p. 168, 1986) and reduces the severity of infections with herpes simplex virus types 1 and 2 in intravaginally infected guinea pigs. In the present report, we evaluated the antiviral activity of S26308 against guinea pig CMV infection in cultured cells. In addition, we assessed the ability of S26308 to limit CMV infection and disease in immunocompetent and immunocompromised guinea pigs.

MATERIALS AND METHODS

Viruses. The prototype strain of guinea pig CMV (CL 22122; American Type Culture Collection, Rockville, Md.) was used. For in vitro studies, a virus stock passaged 12 times in guinea pig embryo (GPE) cells and with a virus infectivity titer of 7×10^6 to 9×10^6 PFU/ml was used. For in vivo studies, virus stocks maintained by serial passage in Hartley guinea pigs were prepared as described previously (10). The virus used in these experiments was at passage number 28 to 31. As needed, samples of virus suspensions stored at −70°C were thawed and diluted to the desired infectivity titer with Hanks buffered saline solution. Vesicular stomatitis virus (VSV) was originally obtained from the American Type Culture Collection (wild type, strain Indiana; ATCC VR158). The virus stock used in the interferon assays had an infectivity titer of 7×10^6 PFU/ml.

Chemical compounds. Compound S26308 was supplied as a free-base powder by Riker Laboratories, St. Paul, Minn. Thomas Matthews (Syntex Research, Mountain View, Calif.) kindly provided DHPG. Compound 2′-fluoro-5-methyl-arabinosyluracil (FMAU) was obtained from Jack J. Fox (The Sloan-Kettering Institute for Cancer Research, Rye, N.Y.). Because of solubility limitations, S26308 was used as a suspension in animal experiments.

Plaque reduction assays. Confluent GPE cell monolayer cultures were grown as described previously (10) in six-well

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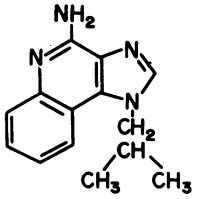

FIG. 1. Chemical structure of S26308.

panels (Costar). Cells were inoculated with approximately 100 PFU of guinea pig CMV per well and incubated at 37°C for 1 h. The cells were then overlaid with Eagle minimal essential medium containing Earle balanced salt solution and 5% heat-inactivated newborn calf serum (MEME–5% DCS) containing 0.5% methylcellulose and appropriate concentrations of the chemical compounds. In each experiment, two to four wells were evaluated for each concentration of antiviral agent and for each set of controls. Cell cultures were incubated at 37°C for 8 days and were fixed and stained with a solution of 10% Formalin containing 1.3% crystal violet. Plaques were enumerated with an inverted microscope. In some experiments, the cell monolayer cultures were pretreated with various concentrations of S26308 for 8 to 72 h prior to virus inoculation.

Cell toxicity assays. Cytotoxicity of S26308 was examined in confluent GPE cell monolayers grown in plastic petri dishes (60 mm diameter). Cells were incubated with MEME–5% DCS containing various concentrations of S26308 for 3 days. Cell counts were performed in duplicate at 24-h intervals by the trypan blue exclusion method. Prior to cell counts, control and drug-treated cells were exposed to phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.02% EDTA for 6 to 8 min and resuspended in MEME–5% DCS.

The effect of S26308 on GPE cell growth was also assessed. GPE cells were seeded into each well of 24-well culture plates and incubated for 24 h at 37°C. Medium was then replaced with MEME–5% DCS supplemented with appropriate concentrations of S26308. At 24-h intervals for 4 days, the number of live cells was determined by the trypan blue exclusion method.

Interferon assay. Serum or cell culture supernatant samples were tested for the presence of interferon as described before (23, 24) with GPE cell monolayers grown in six-well panels and challenged with 50 PFU of VSV. The interferon titer was expressed as the inverse of the highest dilution of serum or supernatant that reduced VSV plaque formation by 50% or more. Supernatants from S26308-treated cultures were obtained as follows. Monolayer cultures of GPE cells were treated with S26308 for 12, 16, or 24 h. The cells were then rinsed three times to remove the compound, fresh medium was added, and the supernatant was collected 4 h later. Serial twofold dilutions of each supernatant sample were assayed in duplicate. In each assay, supernatants of GPE cell cultures not treated with S26308 were included as a negative control. Human leukocyte interferon (20 IU) was also included as a positive control; this concentration resulted in 95% or more reduction of VSV plaque formation.

Animal inoculation and evaluation. Hartley female guinea pigs (250 to 300 g) were purchased from Camm Research Institute (Wayne, N.J.). Prior to virus inoculation, serum samples were collected by cardiac puncture and evaluated for the presence of CMV-neutralizing antibodies as described before (11). Animals without preexisting CMV antibodies were used in this study. The guinea pigs were inoculated subcutaneously in the right axilla with 1 ml of salivary-gland-passaged guinea pig CMV containing 10^5 to 10^6 PFU of virus.

For studies in immunocompetent guinea pigs, four experimental groups were evaluated: (i) the S26308-pretreated and -treated group was given S26308 (3 mg/kg) intraperitoneally (i.p.) daily until the day of sacrifice, starting 1 day prior to virus inoculation; (ii) the S26308-pretreated group was given one single dose of S26308 (3 mg/kg) i.p. 1 day prior to virus inoculation; (iii) the S26308-treated group was injected with S26308 (3 mg/kg) i.p. daily until the day of sacrifice, starting 1 day after virus inoculation; and (iv) the sham-treated group was given PBS i.p. daily until the day of sacrifice, starting 1 day after virus inoculation. Animals were sacrificed on day 7 or 10 after CMV inoculation. To evaluate the toxicity of S26308, some uninfected animals were given S26308 (3 mg/kg) i.p. daily for 8 days.

For studies in immunocompromised guinea pigs, animals were given cyclophosphamide (Cy) i.p. Depending on the experiment, animals received Cy at 30 mg/kg 1 day after virus inoculation, at 30 mg/kg daily for 5 days starting 1 day prior to virus inoculation, or at 300 mg/kg 2 days prior to virus inoculation. In each experiment, guinea pigs received one of the following treatments: (i) the S26308-pretreated and -treated group was given S26308 (3 mg/kg) i.p. daily from 1 day prior to 10 days after virus inoculation; (ii) the S26308-treated group was injected with S26308 (3 mg/kg) daily from 1 to 10 days after virus inoculation; and (iii) the sham-treated group was given PBS daily until 10 days after virus inoculation.

At selected time points, S26308- and sham-treated animals were evaluated for frequency of viremia, hematocrit values, leukocyte counts, and body weights as described before (10, 12, 15). At the time of sacrifice, spleen weights were recorded and tissues were collected for virus isolation and histology as described previously (12, 15). The spleen, lung, and salivary glands were removed aseptically at sacrifice, and virus titrations were carried out by cocultivation on GPE fibroblast monolayers in 24-well panels (10). Cultures were observed for 4 weeks for characteristic CMV-induced cytopathic effect, and viral isolates were identified by specific neutralizing antibodies (12).

RESULTS

Antiviral activity of S26308 against guinea pig CMV infection in vitro. In the first group of experiments, inhibition of guinea pig CMV plaque formation was compared in GPE cell monolayers with drug treatment initiated either 24 h before or 1 h after virus adsorption. S26308 reduced guinea pig CMV plaque formation only when treatment was initiated 24 h prior to virus inoculation (Fig. 2). To determine whether the antiviral activity of S26308 could be enhanced by reducing or prolonging the duration of S26308 pretreatment, we initiated four separate experiments in which cells were treated with S26308 for 8, 16, 20, 24, 48, and 72 h before virus inoculation. Optimal antiviral activity was obtained when cells were treated with S26308 for 16 or 24 h before virus inoculation (data not shown).

Since in the experiments described above S26308 was left in contact with the cells for several days after virus inoculation, we next determined whether the antiviral activity of S26308 remained present if the compound was removed after the pretreatment period. Pretreatment of GPE cells for 24 h prior to virus inoculation resulted in reduction of guinea pig

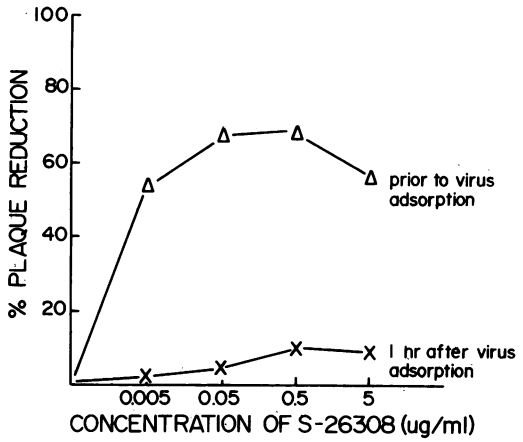

FIG. 2. Inhibition of guinea pig CMV plaque formation in GPE cells by S26308: comparison of S26308 treatment initiated 24 h prior to and 1 h after virus adsorption.

CMV plaque formation whether or not the compound was removed after virus inoculation (Fig. 3).

The ability of S26308 (24-h pretreatment) to reduce plaque formation was compared with that of FMAU and DHPG (Fig. 4). FMAU and DHPG reduced guinea pig CMV plaque formation by 50% at concentrations of approximately 5 and 20 μg/ml, respectively. By contrast, doses of S26308 as low as 0.00005 μg/ml were capable of reducing guinea pig CMV plaque formation by 70% or more. However, in all experiments performed and at all doses of S26308 evaluated, reduction of guinea pig CMV plaque formation remained below 80%.

Investigation of possible mechanisms of antiviral action of S26308 against guinea pig CMV in vitro. The toxicity of various dilutions of S26308, from 0.005 to 500 μg/ml, on confluent GPE cells was examined. Cell toxicity was noted at doses of 500, 50, and 5 μg/ml, but not at doses of 0.5, 0.05, and 0.005 μg/ml (data not shown). The effects of S26308 on GPE cell growth were also determined (Table 1). After 4 days in culture, cell counts were not significantly decreased in cultures incubated with low concentrations of S26308 (0.05 and 0.005 μg/ml) compared with untreated cells.

To ensure that S26308 had no direct virucidal effect, two experiments were performed in which mixtures of guinea pig

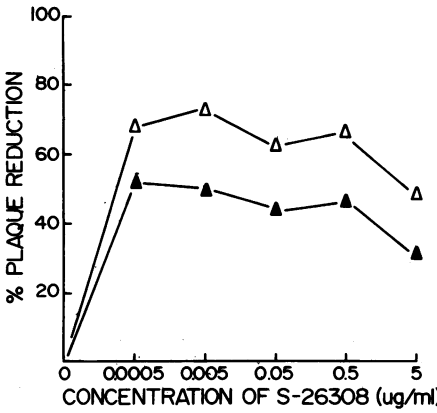

FIG. 3. Inhibition of guinea pig CMV plaque formation following treatment with S26308 24 h prior to virus adsorption: effect of removal of S26308 after virus inoculation. Symbols: △, drug left; ▲, drug removed.

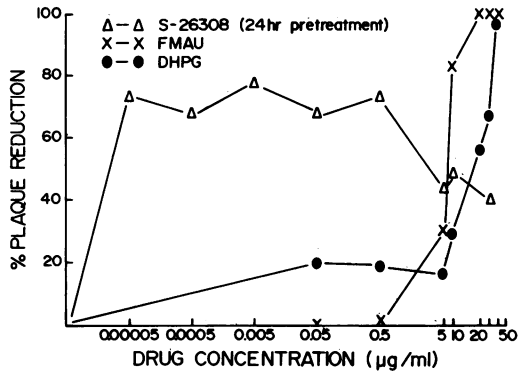

FIG. 4. Inhibition of guinea pig CMV plaque formation by S26308, FMAU, and DHPG.

CMV suspensions and various concentrations of S26308 were incubated at 37 or 25°C for 1 h. Following incubation, virus infectivity titers were determined in untreated and in S26308-treated cultures. Virus infectivity titers were found not to be significantly different in guinea pig CMV suspensions incubated with or without S26308 (data not shown).

To explore another possible mechanism of antiviral action of S26308, we determined whether interferon was induced in GPE cells exposed to S26308. In two separate experiments, supernatants of cell cultures treated with S26308 (0.005 μg/ml) for 12, 16, and 24 h had an interferon titer of 2, 4, and 2, respectively.

Effect of S26308 on acute guinea pig CMV infection in immunocompetent guinea pigs. No significant differences in body weights were noted throughout the course of S26308 administration among the various groups examined. In addition, hematocrit values, total leukocyte counts, and spleen-to-body weight ratios, measured on day 10 after guinea pig CMV inoculation, were similar in all groups of S26308-treated or -pretreated animals and in sham-treated animals. Rates of viremia (Fig. 5) were found to be consistently lower in animals with S26308 treatment initiated 24 h prior to virus administration than in other groups of S26308- or sham-treated animals, except on day 5 after guinea pig CMV inoculation, when all rates were similar.

Virus infectivity titers in the spleen, lung, and salivary glands obtained from the four groups of animals were also evaluated (Table 2). Differences among the various groups examined were not significant. By contrast, results of the histological evaluation indicated differences between experimental groups (Table 3). Although animals in the three groups showed similar extents of histopathology, inclusions were not seen in the spleens of animals in the S26308-pretreated and -treated group but were seen in 75 and 67% of animals in the S26308-treated and sham-treated groups,

TABLE 1. Effect of S26308 on GPE cell growth[a]

S26308 concn (μg/ml)	Live cells (% of control untreated cells) on day:			
	1	2	3	4
5	79	82	80	87
0.5	97	114	100	81
0.05	96	111	93	100
0.005	94	104	103	96

[a] S26308 was added to GPE cells on the day of cell seeding. Live cells were enumerated by the trypan blue exclusion method. At each concentration and for each day, four wells were evaluated.

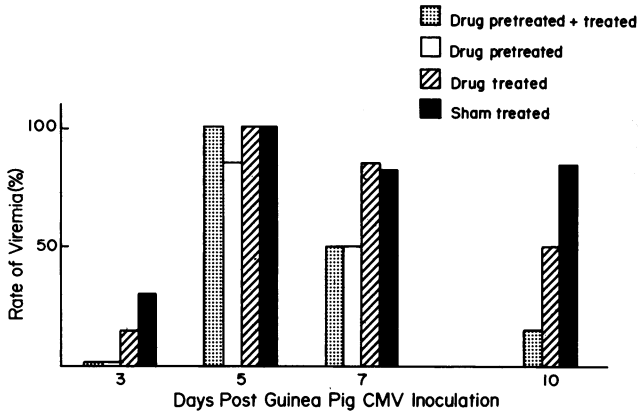

FIG. 5. Effect of S26308 on the rate of viremia during acute guinea pig CMV infection in immunocompetent guinea pigs.

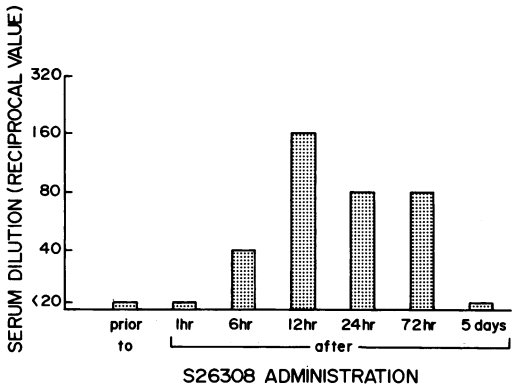

FIG. 6. Detection of interferon in guinea pig serum at different times after a single administration of S26308. Results are expressed as the inverse value of the highest dilution that produced at least 50% reduction of VSV plaque formation.

respectively, examined on day 7 post-guinea pig CMV inoculation. Uninfected guinea pigs given S26308 i.p. for 8 days had no histological evidence of drug-induced toxicity.

To determine whether interferon could be detected in S26308-treated guinea pigs, sequential serum samples were obtained following one injection of the compound. The results obtained in one of two experiments performed are shown in Fig. 6. Peak interferon levels were detected 12 h postinoculation of S26308. Interferon was no longer detectable in serum 5 days after S26308 administration.

Effect of S26308 on acute guinea pig CMV infection in immunocompromised guinea pigs. Three experiments were performed to evaluate the therapeutic efficacy of S26308 in guinea pigs immunocompromised with Cy. In one experiment, animals were given Cy (30 mg/kg) for 5 days, starting 1 day prior to administration of 10^5 PFU of virus (Table 4). Deaths occurred in the sham- and S26308-treated groups only, and all animals in both these groups were found to be viremic. By contrast, no deaths occurred in the group pretreated and treated with S26308, and 66% of these animals were viremic on day 7. In addition, weight gains were significantly higher in this group than in the sham-treated group. In a second experiment, animals inoculated with 5×10^5 PFU of virus were given one injection of Cy (30 mg/kg) 1 day prior to virus inoculation. Body weight gains were higher and rates of viremia were lower in S26308-pretreated and -treated than in sham-treated guinea pigs. However, death rates were similar in both groups. In a third experiment (data not shown), animals were given one single dose of Cy (300 mg/kg) 2 days prior to inoculation with 10^5 PFU of virus. There was no difference among the S26308-pretreated and -treated, S26308-treated, or sham-treated animals. All animals had severe lethal infections, and viremia was demonstrated in all animals evaluated.

DISCUSSION

These results show that Riker compound S26308 had an antiviral effect against guinea pig CMV infection in vitro. Guinea pig CMV plaque formation was reduced only in GPE cells treated with S26308 prior to virus infection. The same concentration of compound had no effect when added to GPE cells after virus absorption. Treatment of GPE cells for 16 to 24 h prior to virus adsorption was most efficacious in reducing CMV plaque formation. The antiviral effect remained even when the compound was removed after virus adsorption. Compared with DHPG and FMAU, much lower concentrations of S26308 (0.00005 μg/ml) were capable of reducing guinea pig CMV plaque formation. FMAU and DHPG were found to reduce guinea pig CMV plaque formation by 50% at concentrations similar to those used previously (8, 9). It is important to note that at all doses of S26308 evaluated, we could not demonstrate complete inhibition of guinea pig CMV. Reduction of guinea pig CMV plaque formation by S26308 remained below 80%, the dose response remained flat, and protection was found to be only partial.

The antiviral effect in vitro was due to neither direct virucidal activity nor inhibition of GPE cell proliferation. We detected limited amounts of interferon in supernatants of S26308-treated cells. This suggests that the antiviral effect

TABLE 2. Effect of S26308 on virus infectivity titers in guinea pig tissues at 7 and 10 days postinoculation of immunocompetent guinea pigs with guinea pig CMV

Group[a]	Mean virus infectivity titer ($\log_{10}$ $TCID_{50}$/0.01 g of tissue)[b] ± SE					
	Day 7			Day 10		
	Spleen	Lung	Salivary glands	Spleen	Lung	Salivary glands
S26308 pretreated and treated	2.41 ± 0.90	2.32 ± 1.06	1.52 ± 0.64	1.37 ± 0.65	1.62 ± 0.79	4.26 ± 1.88
S26308 pretreated	2.77 ± 1.27	1.83 ± 0.85	1.45 ± 0.65	ND[c]	ND	ND
S26308 treated	2.62 ± 1.18	1.75 ± 0.75	2.28 ± 1.04	1.67 ± 0.67	1.15 ± 0.37	3.26 ± 1.47
Sham treated	3.18 ± 1.30	1.58 ± 0.64	1.87 ± 0.79	1.88 ± 0.85	1.25 ± 0.77	3.79 ± 1.60

[a] Six animals were examined on each day for each group.
[b] $TCID_{50}$, 50% tissue culture infective dose.
[c] ND, Not done.

TABLE 3. Effect of S26308 on histopathology[a] in guinea pig tissues 7 and 10 days postinoculation of immunocompetent guinea pigs with guinea pig CMV

Group	Day 7			Day 10	
	% of animals with inclusions in spleen	% of tissues with:		% of tissues with:	
		Inclusions	Histopathology	Inclusions	Histopathology
S26308 pretreated and treated	0	15	70	33	61
S26308 treated	75	37	68	28	67
Sham treated	67	27	80	29	64

[a] Tissues examined included lung, liver, spleen, kidney, salivary glands, and bone marrow. For each group, 36 tissue samples were examined. Tissues of six uninfected guinea pigs given S26308 i.p. (3 mg/kg per day) for 8 days showed no evidence of drug toxicity.

observed may be due to interferon induction by S26308. The fact that only low levels of anti-VSV activity were detected is not surprising. Interferon from primary GPE cells has atypical characteristics and requires special treatment to preserve full antiviral activity (24). Previous studies have found that guinea pig cells infected by several different viruses are capable of producing only low levels of interferon (4, 20, 22). Winship et al. have shown that guinea pig interferon is more sensitive to heat and pH changes than mouse interferon. Thus, it is very labile and difficult to detect by conventional methods (23, 24).

The in vitro activity of S26308 against guinea pig CMV infection was reflected in vivo in both immunocompetent and immunocompromised guinea pigs. Timing of the administration of S26308 in relation to guinea pig CMV infection was important to the efficacy of the compound. Rates of viremia were found to be most reduced in guinea pigs that had S26308 therapy initiated 24 h prior to virus inoculation. In immunocompetent guinea pigs, the reduced level of viremia was accompanied by some level of protection in the spleens of prophylactically treated animals, since inclusions were not seen in the spleens of animals that had S26308 therapy initiated 24 h prior to virus inoculation but were present in other animals. This reduction in the severity of splenic involvement in prophylactically treated animals did not correlate with a reduction in virus infectivity titers in the spleen. In immunocompromised guinea pigs, the reduction in the extent of viremia, albeit modest, was accompanied by modulation of a severe lethal infection to a significantly less severe clinical infection. The present studies with immunocompromised animals underline the need for careful choice and monitoring of the virus inoculum and Cy doses. Indeed, depending on the severity of the acute CMV infection induced, the efficacy of S26308 could be demonstrated to vary. This model of severe generalized guinea pig CMV infection may be relevant and useful for the evaluation of antiviral agents in immunocompromised hosts.

Morbidity and mortality due to CMV are most likely to develop in transplant recipients and in patients with acquired immunodeficiency syndrome. Prophylactic administration must be considered when planning strategies for prevention of human CMV infections in these immunocompromised patients. Prophylactic use of interferon in renal transplant recipients has met with some success. Cheeseman et al. (3) reported a decreased rate of viremia and a delay in CMV excretion after transplantation in patients who received 6 weeks of leukocyte interferon (alpha interferon) starting on the day of transplantation. In seropositive renal transplant recipients, alpha interferon afforded effective prophylaxis against serious CMV infection (13). As reported before, we found that guinea pigs given S26308 i.p. had detectable levels of interferon in their serum. Prophylactic and therapeutic use of S26308 resulted in reduction of the extent of CMV viremia in guinea pigs. Although this reduction did not prevent virus spread to target tissues, it did result in some reduction of the severity of CMV infection. Future evaluations of S26308 should include alternative dose regimens and routes of administration, considering pharmacokinetic data and levels of interferon induced in vivo. Studies with bone marrow transplant recipients have indicated that CMV viremia occurs several weeks before CMV pneumonia develops (16). Since patients with clinical evidence of diffuse CMV-induced interstitial pneumonia are least likely to respond to antiviral therapy (5), antiviral therapy directed toward interrupting the progression from CMV viremia to interstitial pneumonia and death may be successful in these patients.

TABLE 4. Effect of S26308 on the severity of acute guinea pig CMV infection in immunocompromised guinea pigs

Cy dose (mg/kg) and regimen	Virus inoculum (PFU)	Group[a]	Mortality		Mean wt change[b] (% of wt on day 0) ± SD	Viremia[b] (% of animals)
			No. dead/ no. tested	Mean day of death (day post-CMV inoculation)		
30, 5 days	1×10^5	S26308 pretreated and treated	0/4		105.8 ± 2.9	66
		S26308 treated	2/5	16	103.2 ± 5.4	100
		Sham treated	2/5	14	98.3 ± 3.8	100
30, 1 day	5×10^5	S26308 pretreated and treated	2/6	12	99.4 ± 3.1	50
		Sham treated	1/4	10	96.2 ± 3.1	100

[a] All animals were treated until day 10 post-CMV inoculation.
[b] Evaluated on day 7 post-CMV inoculation.

The efficacy of S26308 as a prophylactic compound that can reduce the extent of CMV viremia and prevent severe CMV disease might be improved if S26308 is used in combination with other therapeutic antiviral agents.

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