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Studies on the effect of CL 306,293, a substituted quinoline carboxylic acid, on the clinical disease induced in mice with LP-BM5 virus

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Summary

CL 306,293, a substituted quinoline carboxylic acid, is a potent inhibitor of dihydroorotic acid dehydrogenase, an enzyme essential for the biosynthesis of pyrimidines. In mammalian cell culture, the agent exhibits antiproliferative properties that can be reversed by the addition of uridine. CL 306,293 inhibits the development of the clinical disease in a murine model of immunodeficiency induced by a mixture of LP-BM5 retroviruses. In infected mice, the agent prevents the development of hypergammaglobulinemia, lymphadenopathy, splenomegaly and induction of an IL-2 deficiency. The CD_4/CD_8 ratio and the number of B cells in the lymph nodes are decreased if the infected animals are treated with CL 306,293. CL 306,293 was more efficacious and potent than 3′-azido-3′-deoxythymidine. The beneficial effects of CL 306,293 observed in this model are most probably related to its antiproliferative properties.

MAIDS; AIDS; LP-BM5 retrovirus; MuLV; CL 306,293; Antimetabolite; Dihydroorotic acid dehydrogenase inhibitor; Antiproliferative; Azidothymidine

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Introduction

Infection of certain strains of mice with a mixture of LP-BM5 murine retroviruses leads to a severe immunodeficiency called murine acquired immunodeficiency syndrome (MAIDS). The clinical disease is manifested by progressive lymphadenopathy, splenomegaly, hypergammaglobulinemia, increased susceptibility to infections, B-cell lymphomas and profound immunosuppression. Since some of these lesions resemble those associated with AIDS, the LP-BM5 murine model has been considered to be a potential model of the human disease (Jolicoeur, 1991).

The pathogenesis of the LP-BM5 disease has been extensively studied. The disease is MHC linked and mice that bear the haplotype H_2^d are disease resistant (Makino et al., 1990; Hamelin et al., 1989). Polyclonal expansion of T- and B-cells in the lymphoid organs has been observed. Cell-sorting analysis has shown that there is a selective depletion of $CD8^+$ cells and a progressive increase in $CD3^+$ cells that are Thy-1^- (Holmes et al., 1990; Morse et al., 1989). This progressive increase in B-cells leads to B cell lymphomas. Athymic mice or mice depleted of $CD4^+$ T-cells do not develop the lesion (Mosier et al., 1987; Yetter et al., 1988). In addition, depletion of B-cells using anti-mouse IgM antibody prevents the immunosuppression induced by the LP-BM5 virus that suggests that both B- and T-cells are required for the induction of the clinical disease.

The role of virus replication has also been investigated (Huang et al., 1989). These studies have shown that virus replication is not a prerequisite for the induction of clinical disease. Instead, the virus infects unidentified target cells and immunosuppression results from proliferation of these target cells. Consistent with this hypothesis, recent studies have demonstrated that cyclophosphamide, a cytotoxic agent, can attenuate the clinical disease induced by the LP-BM5 virus. In spleens derived from mice infected with the virus and treated with cyclophosphamide, viral DNA was absent (Simard et al., 1991). 3′-azido-3′-deoxythymidine (AZT) can also attenuate the disease, if administered at the time of viral infection (Ohnota et al., 1990; Eiseman et al., 1991).

CL 306,293 (Fig. 1), a substituted quinoline carboxylic acid, is a potent immunomodulatory agent that modulates T-cell function (Sloboda et al., 1991). Mechanistic studies have demonstrated that this agent is a potent inhibitor of mammalian dihydroorotic acid dehydrogenase, an enzyme essential for the biosynthesis of uridine and cytidine. Consistent with this observation, growth of mammalian cells was inhibited by CL 306,293 and this growth inhibition was completely reversed upon the addition of uridine, but not cytidine, to the growth medium. When mice infected with LP-BM5 virus were treated orally with CL 306,293, the clinical disease associated with the virus infection was attenuated. Results of a comparative study suggest that CL 306,293 was more potent and more efficacious than AZT. Daily treatment with CL 306,293 was required and if treatment was suspended, a relapse of the

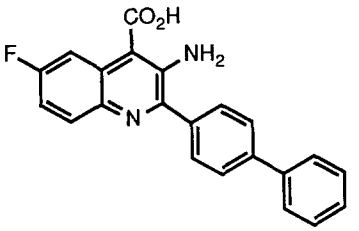

Fig. 1. Structure of CL 306,293.

clinical disease was observed. Progression of the disease has also been observed when treatment with AZT has been suspended (Eiseman et al., 1991). Thus, treatment with either agents does not induce long-term cures.

Materials and Methods

C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, ME or from Charles River, Wilmington, MA, and used in these studies. Cell-free virus stock of LP-BM5 MuLV ($10^5 \times$ c PFU/ml of ecotrophic MuLV and 10^4 MCF MuLV focus-forming units/ml) was prepared by methods already described (Mosier et al., 1985; Laterjet et al., 1962). Mice were injected intraperitoneally with 0.25 ml of the cell-free virus stock solution. Immediately upon inoculation, the mice were treated orally by gavage with various doses of CL 306,293, or AZT dissolved in saline. The mice were treated daily for 28 days. In some studies with CL 306,293, treatment was suspended in infected mice at 28 days and the mice maintained for an additional 7 days. In other studies normal uninfected mice were treated daily with various oral doses of CL 306,293. After 28 days of treatment, the mice were killed and the spleen and lymph nodes removed for analysis.

In most studies, the mice were bled on day 28 and the serum analyzed for IgM content by a standard ELISA procedure. Briefly, wells of microtiter plates were coated overnight at 4°C with 0.2 ml of goat anti-mouse IgM (1 μg/ml; Southern Biotechnology Associates, Birmingham, AL) diluted in PBS. Unblocked sites were blocked by the addition of 10% fetal calf serum diluted in PBS. After 1 h at room temperature, the wells were washed with PBS and incubated with various dilutions of mouse serum diluted in PBS containing 1% BSA and 0.1% Tween-20®. After incubation at room temperature for 3 h, the wells were washed and incubated with alkaline phosphatase conjugated to goat anti-mouse κ (0.4 μg/ml of PBS containing 1% BSA and 0.1% Tween-20®; Southern Biotechnology Associates). After 3 h at room temperature, the wells were washed and incubated with *p*-nitrophenyl phosphate (0.3 mg/ml). After 30 min, the plate was read in an ELIDA® spectrophotometer (Physica Inc., New York, NY). Various concentrations of murine IgM were used as a standard in each assay and control wells contained PBS.

Single-cell suspensions of spleens and lymph nodes were prepared. The cell content was determined using a Coulter® counter. 1 ml of the spleen cell preparation (1×10^7 cells/ml) was incubated in DMEM (GIBCO) supplemented with 5% fetal calf serum, L-glutamine, penicillin/streptomycin and concanavalin A (5 μg/ml) for 18 h. The supernatant was assayed for IL-2 using CTLL-2 cells (Gillis et al., 1978; Schmitt et al., 1983). Lymph-node cells were stained with PE-anti-Lyt2, FITC-anti-L3T4 (Becton Dickinson & Co.) and biotin-goat anti-μ streptavidin-PE (Tago Immunologicals) and analyzed by flow cytometry (FACS IV System, Becton Dickinson & Co.). Each antibody preparation was titrated to obtain maximal staining and capping was reduced by keeping cells at 4°C (DeJoy et al., 1989).

A highly purified preparation of dihydroorotic acid dehydrogenase was a gift of Dr. M. Johnston, University of Chicago. The effect of CL 306,293 on the activity of this enzyme was assayed by a spectrophotometric method. Details of this assay have been published (Hines et al., 1989a; Hines et al., 1989b).

To determine the effects of CL 306,293 on the growth of L-929 cells, replicate cultures (1×10^6 cells/ml) were exposed for 24 h to various concentrations of the agent. 6 h before harvest, the cultures were exposed to radiolabeled leucine. The amount of radioactivity incorporated was measured by lysing the cells and analyzing for hot trichloroacetic-acid-precipitable radioactivity. In some studies, cold uridine or cytidine was added to cell cultures containing CL 306,293.

Results

The effect of various concentrations of CL 306,293 on the enzymatic activity of dihydroorotic acid dehydrogenase is shown in Fig. 2. Using the standard Dixon plot, the intercept at the x-axis indicates a K_i of approx. 13 nM. Thus, CL 306,293 is a potent inhibitor of dihydroorotic acid dehydrogenase.

The effect of CL 306,293 on the growth of L-929 cells was also investigated. Results shown in Fig. 3 indicate that CL 306,293 inhibits the growth of L-929 fibroblasts in a dose-dependent manner. In results not shown, addition of 100 μM uridine, but not cytidine, completely reversed the growth inhibition observed with CL 306,293. These observations are consistent with the view that CL 306,293 inhibits cell growth due to depletion of intracellular uridine and growth is restored if the cells are supplied with exogenous uridine. Exogenous cytidine is not able to restore growth because of the inability of these cells to convert cytidine to uridine by way of a cytidine deaminase.

A representative experiment comparing the effect of CL 306,293 and AZT on the clinical disease induced by LP-BM5 virus is shown in Table 1. Confirming earlier observations (reviewed by Jolicoeur, 1991) infected mice exhibited hypergammaglobulinemia (IgM), lymphadenopathy, splenomegaly and a deficiency of splenic IL-2 synthesis. If these mice were treated with CL 306,293 (12.5 to 3.1 mg/kg), serum IgM content was significantly lower. The

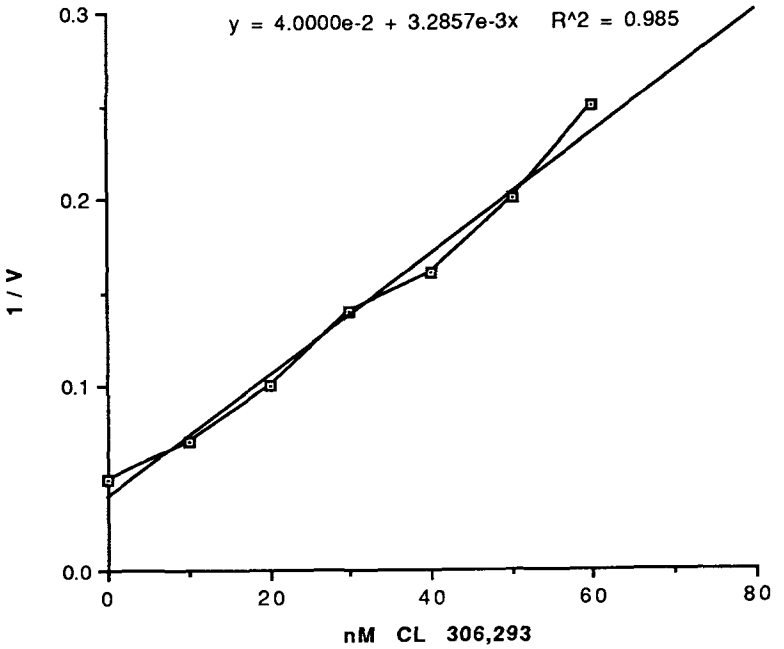

Fig. 2. Effect of various concentrations of CL 306,293 on the enzymatic activity of dihydroorotic acid dehydrogenase. The velocity of the reaction was measured as change in absorbance at 600 nm/min per unit enzyme. Regression analysis shown in the figure indicates an inverse correlation between the concentration of CL 309,293 and enzyme velocity.

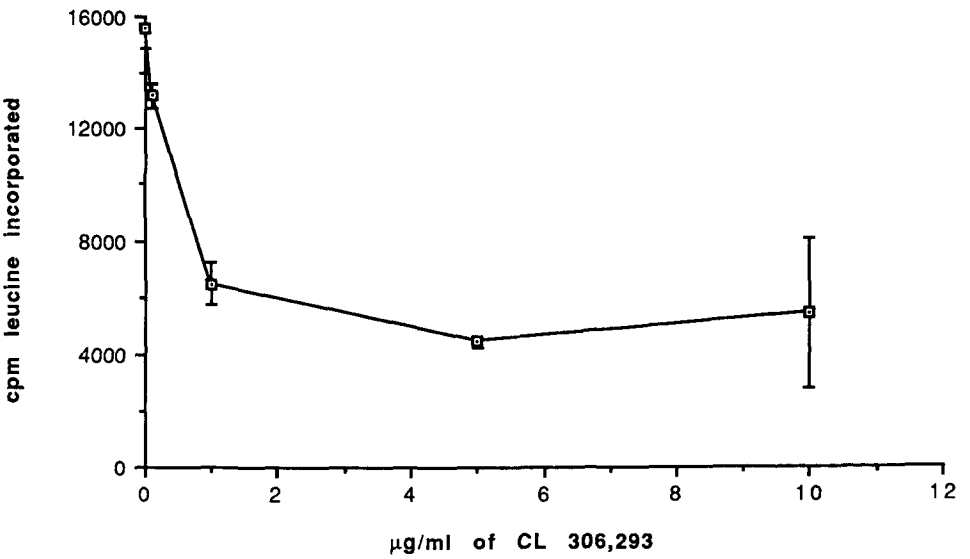

Fig. 3. Effect of various concentrations of CL 306,293 on leucine incorporation by L-929 fibroblasts.

TABLE 1

The effect of various doses of CL 306,293 and AZT on the clinical disease associated with LP-BM5 infection

Treatment	dead/ treated	Serum IgM (μg/ml)	Mean ± S.E. cells ($\times 10^6$)		IL-2 synthesis (units/ml)
			spleen	lymph node	
normals	0/10	802 ± 81	105 ± 7	4.8 ± 0.5	267 ± 23
LP-BM5 infected	0/10	4621 ± 516*	207 ± 13*	12.8 ± 0.8*	2 ± 1.7*
+ CL 306,293					
12.5 mg/kg	0/10	614 ± 80**	127 ± 15**	2.6 ± 0.6[a]	138 ± 36**
6.2 mg/kg	0/10	847 ± 99**	117 ± 4**	5.4 ± 0.6[a]	204 ± 27**
3.1 mg/kg	0/10	1076 ± 83**	144 ± 4**	7.7 ± 0.7[a]	123 ± 15**
LP-BM5 infected					
+ AZT					
200 mg/kg	7/10	3139 ± 952	134 ± 22[a]	6.4 ± 0.2[b]	106 ± 71**
100 mg/kg	0/10	4556 ± 339	166 ± 4[a]	8.9 ± 1.1[b]	32 ± 13[b]
50 mg/kg	0/10	4502 ± 690	171 ± 16	8.7 ± 0.8**	36 ± 16

Mice were killed at day-28 post-infection and treatment. *$P < 0.001$ as compared to normals; **$P < 0.001$ as compared to LP-BM5 controls; [a]$P < 0.02$ as compared to LP-BM5 controls; [b]$P \leq 0.05$ as compared to LP-BM5 controls (Student's t-test). All of the survivors were used for the analysis shown. Other details of the assay are described in the text.

cell content of the spleen and lymph nodes was also lower in mice treated with CL 306,293. Splenocytes from normal, infected and those treated with CL 306,293 were stimulated with concanavalin A and the IL-2 content in the medium was determined. Results shown in Table 1 indicate in infected mice treated with 12.5 to 3.1 mg/kg of CL 306,293, IL-2 content was significantly higher than in infected control mice. In results not shown, the agent was inactive at a dose of 1.5 mg/kg.

The effect of AZT on the clinical disease associated with LP-BM5 infection is also shown in Table 1. Surprisingly, significant mortality was observed (during week 3 of treatment) when the agent was administered as a bolus dose of 200 mg/kg. Earlier studies have demonstrated that AZT at 250 mg/kg suppresses clinical disease associated with LP-BM5 infection if the agent is administered in drinking water (Ohnota et al., 1990). Thus the increased toxicity associated with AZT observed in our studies is most probably related to the difference in the treatment regimen. Nevertheless, in the surviving mice, splenomegaly and lymphadenopathy was significantly suppressed by AZT at a dose of 200 mg/kg. The ability of splenocytes to synthesize IL-2 was significantly higher. At a dose of 100 mg/kg, serum IgM levels were no different from those values observed for infected control mice. However, splenomegaly and lymphadenopathy was suppressed by AZT at this dose. IL-2 synthesis by splenocytes was significantly higher. At 50 mg/kg, serum IgM levels, IL-2 synthesis and splenomegaly were unaffected but lymphadenopathy was significantly suppressed.

To ascertain whether the effects of CL 306,293 observed in Table 1 were due to toxic effects of the agent, normal mice were treated with various doses of CL 306,293. On day 28, the mice were sacrificed and the plasma analyzed for IgM.

TABLE 2

The effect of CL 306,293 in normal mice

Treatment	dead/ treated	Serum IgM (μg/ml)	Mean ± S.E. cells ($\times 10^6$)		IL-2 synthesis (units/ml)
			spleen	lymph node	
None	0/5	1080 ± 78	114 ± 23	5.1 ± 0.8	230 ± 47
CL 306,293					
12.5 mg/kg	0/5	1010 ± 43	103 ± 31	5.7 ± 0.6	219 ± 39
6.25 mg/kg	0/5	1133 ± 64	96 ± 41	5.4 ± 0.9	262 ± 59
3.1 mg/kg	0/5	1142 ± 57	110 ± 31	5.2 ± 0/4	228 ± 43

Mice were treated daily for 28 days orally with CL 306,293 at doses indicated. On day 28, they were bled and killed. The data shown are derived from 5 mice per group. Other details of the assay are described in the text.

The cell content of the spleen and lymph nodes was also ascertained. Splenocytes were stimulated with concanavalin A and the supernatant analyzed for IL-2. Results shown in Table 2 indicate that the agent had no effect on the various parameters that were measured. These observations suggest that CL 306,293 at the doses tested had no deleterious effect on the immune system.

The lymph node cells of (a) normal mice (b) those infected with LP-BM5 and (c) those infected with the virus and treated with CL 306,293 were stained with monoclonal antibodies used for the identification of CD4-, CD8- and B-cells. The stained cells were analyzed by flow cytometry. The results of these studies are shown in Table 3. In lymph nodes of infected mice, there was a depletion of $CD8^+$ T-cells, an increase in the ratio of $CD4^+/CD8^+$-bearing T-cells and an increase in the number of B-cells. In lymph nodes of mice treated with CL 306,293 and AZT, the percentage of cells bearing the CD8 marker was significantly higher and the CD4/CD8 ratio was significantly lower. In addition, the number of B-cells in the lymph node of infected mice treated

TABLE 3

Phenotypic analysis of lymph nodes of LP-BM5 infected mice treated with CL 306,293 or AZT

treatment	n	percent $CD8^+$ cells	Mean ± S.E. $CD4^+/CD8^+$ ratio	B-cells $\times 10^6$
normals	10	18.5 ± 2.3	1.5 ± 0.1	1.5 ± 0.3
LP-BM5 infected		7.6 ± 0.8*	2.6 ± 0.2*	6.5 ± 0.5
+ CL 306,293				
12.5 mg/kg	10	25.0 ± 2.4**	1.1 ± 0.1**	1.0 ± 0.5**
6.2 mg/kg	10	15.6 ± 0.5**	1.4 ± 0.1**	1.9 ± 0.2**
3.1 mg/kg	10	19.3 ± 0.9**	1.4 ± 0.1**	2.7 ± 0.3**
+ AZT				
200 mg/kg	3	15.3 ± 0.3**	1.5 ± 0.1**	3.4 ± 1.2**
100 mg/kg	10	12.1 ± 2.3**	1.7 ± 0.2**	3.9 ± 0.8**
50 mg/kg	10	17.8 ± 3.5**	1.3 ± 0.1**	4.3 ± 0.6**

Mice were killed on day-28 post infection and treatment. *$P < 0.05$ as compared to normals; **$P < 0.05$ as compared to LP-BM5 infected mice (student's t-test). n = number of mice used for analysis. Other details of the assay are described in the text.

TABLE 4

Cessation of treatment with CL 306,293 (on day 28) of LP-BM5 infected mice causes an increase in serum IgM levels (day 35 observation)

	Serum IgM levels (μg/ml $\pm$ S.E.)	
	day 28	day 35
normals	321 $\pm$ 51	285 $\pm$ 30
LP-BM5 infected	732 $\pm$ 107*	892 $\pm$ 179*
+ CL 306,293		
12.5 mg/kg	493 $\pm$ 52**	1283 $\pm$ 349
6.2 mg/kg	298 $\pm$ 39**	1037 $\pm$ 218
3.1 mg/kg	282 $\pm$ 38**	844 $\pm$ 244

5 mice used for each analysis. *$P < 0.05$ as compared to normals; **$P < 0.05$ as compared to LP-BM5 infected controls. Other details of the assay are described in the text. Note that C57/BL6 mice from Charles River were used in this experiment. The difference in serum IgM levels (this table and Table 1) may be due an increased severity of the disease in the mice supplied by Jackson Laboratories. Nevertheless, treatment with CL 306,293 caused a significant decrease in serum IgM levels and upon suspension of treatment, serum IgM levels were increased.

with CL 306,293 or AZT was significantly lower.

To determine whether suspension of treatment with CL 306,293 would affect the progression of the disease in treated mice, infected mice were treated daily with CL 306,293 for 28 days. The mice were bled for serum IgM analysis and treatment suspended. Seven days after cessation of treatment, mice were bled again, the serum IgM content analyzed and used as an indicator of disease progression. Results shown in Table 4 indicate that upon cessation of treatment, serum IgM content increased significantly suggesting that continuous treatment with CL 306,293 is required to control the clinical disease.

Discussion

The demonstration of the biological activity of cyclophosphamide in the LP-BM5 murine model of immunodeficiency (Simard et al., 1991) has generated considerable interest in evaluating immunosuppressive or immunomodulatory agents in animal models of AIDS. If clinical activity is detected in this animal model, further evaluation of these agents can be conducted in other relevant models. The main advantage of the MAIDS model is that it does not require extensive animal containment facilities and agents that show activity in this model can be considered for further preclinical and clinical development.

CL 306,293 is a new antiproliferative agent that modulates T-cell function and has been shown to exhibit antiinflammatory and antiarthritic properties in animal models of arthritis and graft versus host disease (Sloboda et al., 1991). This agent was chosen for evaluation in the MAIDS model.

The present studies demonstrate that CL 306,293 inhibits the development of clinical disease induced in mice by the mixture of LP-BM5 retroviruses and that

it is more potent and efficacious than AZT. Similar to AZT, discontinuation of therapy with CL 306,293 induces a relapse of the clinical disease; thus, treatment with CL 306,293 is not curative. The mechanism by which CL 306,293 inhibits the development of the clinical disease induced by LP-BM5 virus is most probably related to its antiproliferative effects (inhibition of dihydroorotic acid dehydrogenase). The agent does not induce interferon and as expected of an antimetabolite, anti-SRBC or anti-ovalbumin responses in mice are suppressed when immunized mice are treated with the agent (results not reported). Recent studies (Hugin et al., 1991) have suggested that $Pr60^{gag}$, the protein encoded by the LP-BM5 virus is a superantigen that can induce non specific activation of T-cells. Subsequent release of lymphokines by these activated T-cells could lead to proliferation of lymphoid cells leading to lymphadenopathy, splenomegaly and a profound immunodeficiency. CL 306,293 may inhibit the T- and B-cell proliferation induced by $Pr60^{gag}$ and thereby inhibit the development of clinical disease induced by the mixture of LP-BM5 viruses. In this context, it should be noted that cyclosporin, an immunosuppressive agent can also inhibit the development of clinical disease induced by LP-BM5 virus (Cerny et al., 1991). Thus, the suppression of the clinical disease by CL 306,293 is probably related to its antiproliferative effects rather than an antiviral effect as observed with AZT. Preliminary studies conducted with CL 306,293 support this view. For example, when CL 306,293 is added to MT-2 T-cells (human) that are infected with cytopathic concentrations of HIV IIIB, the agent does not protect them from the cell kill induced by HIV IIIB. In this assay, cell kill was analyzed by the standard MTT dye assay. Since CL 306,293 is an antiproliferative agent, this result is not surprising and additional studies were conducted with H9 cells infected with HIV IIIB. In these studies the amount of reverse transcriptase (RT) in the medium was assayed after a 7 day incubation. In media derived from infected cells incubated with CL 306,293 (10 and 1 μg/ml), the amount of RT in the medium (assayed by the method of Poiesz et al., 1980) was decreased by 95%. Parallel studies indicate that at similar concentrations, the agent was cytotoxic. Thus, the reduction in the amount of RT in the medium was related to the cytotoxicity of CL 306,293. In other screening studies related to human cytomegalovirus and herpes simplex virus 1, CL 306,293 did not exhibit antiviral properties.

Although the pathogenesis of MAIDS is different from AIDS, the MAIDS model can still be used as a prescreen for antiviral agents that may potentially be useful in the treatment of AIDS (Gangemi et al., 1989). The model is reproducible and surrogate markers of the clinical disease can be readily measured. When a large number of agents need to be evaluated, the MAIDS model is the model of choice. Other animal models of AIDS (Namikawa et al., 1988; Kaneshima et al., 1991; Gardner, 1989; Desrosiers et al., 1989) are not cost effective and require extensive containment facilities. Since CL 306,293 is active in the MAIDS model, evaluation in other animal models more relevant to AIDS need to be conducted to ascertain its activity in AIDS.

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