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Inhibition of Murine Hepatitis Virus Infections by the Immunomodulator 2,3,5,6,7,8-Hexahydro-2-Phenyl-8,8-Dimethoxy-Imidazo[1,2a]Pyridine (PR-879-317A)

R. W. SIDWELL,[1]* J. H. HUFFMAN,[1] E. W. CALL,[1] R. P. WARREN,[2] L. A. RADOV,[3] AND R. J. MURRAY[3]

Department of Animal, Dairy and Veterinary Sciences[1] and Department of Biology,[2] Utah State University, Logan, Utah 84322-5600, and Department of Pharmacology, Pharmaceuticals Division, Pennwalt Corp., Rochester, New York 14623[3]

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PR-879-317A (2,3,5,6,7,8-hexahydro-2-phenyl-8,8-dimethoxy-imidazo[1,2a]pyridine) has been found to be a T-cell-selective immunomodulating agent. In the current studies, a series of experiments was designed to determine the potential antiviral activity of this compound in mice infected with murine hepatitis virus. In a comparative antiviral experiment, the activity seen was superior to that of levamisole, a known immunorestorative agent. This activity was characterized by an increase in the 21-day survival frequency, a decrease in hepatic discoloration, a decrease in the amount of infectious virus recoverable from the liver, and normalization of serum glutamic oxalacetate and pyruvate transaminase levels. A comparison of treatment routes indicated the relative efficacies as intraperitoneal > per os > intramuscular ≥ subcutaneous. Alteration of the treatment schedule markedly affected the antiviral effect; prophylactic or therapeutic treatments once or twice daily for 3 days were usually effective. Single treatments begun 4 h before or 24 h after virus inoculation were highly efficacious. Three treatments administered on alternate days, beginning 48 h before virus inoculation, proved moderately effective. Thrice-daily treatments were ineffective, as were treatments with durations of greater than 3 days. The optimal dosage varied according to the treatment route and dosage schedule. When assessed for direct antiviral activity in vitro, PR-879-317A failed to demonstrate any significant activity against murine hepatitis virus. The positive in vivo activity noted might therefore be the result of immune modulation rather than a direct antiviral effect.

A major strategy in the treatment of viral infection is to bolster the immunological resistance of the host. Early efforts in this regard included the use of interferon inducers (5) and the live bacillus Calmette-Guerin (6) to enhance natural resistance to viral infections. A potential advantage to such approaches has been the prospect of developing selective agents that have a broad spectrum of antiviral effect but are devoid of the side effects often associated with classical antiviral drugs. Although a number of immunomodulatory agents have been evaluated in both human and animal viral diseases (for a review, see reference 15), none has previously proven to be sufficiently efficacious against viral infections.

PR-879-317A (2,3,5,6,7,8-hexahydro-2-phenyl-8,8-dimethoxy-imidazo[1,2a]pyridine), whose structure is shown in Fig. 1, has been developed as a selective humoral and cellular immunorestorative agent (L. A. Radov, R. J. Murray, and C. R. Kinsolving, Int. J. Immunopharmacol., in press; R. P. Warren, M. C. Healey, A. V. Johnston, R. W. Sidwell, L. A. Radov, and R. J. Murray, Int. J. Immunopharmacol., in press). The present report describes results of a series of studies designed to delineate the potential antiviral properties of this compound. Specifically, these studies investigated the effects of PR-879-317A on mice infected with murine hepatitis virus (MHV), a coronavirus.

MATERIALS AND METHODS

Virus. The original (Friend-Braunsteiner) strain of MHV, obtained from the American Type Collection (ATCC, Rockville, Md.), was used in these studies. For in vivo passage of the virus, it was initially injected intraperitoneally (i.p.) into specific-pathogen-free Swiss Webster mice. Five days later, the mice were killed and their livers were removed for preparation of a 10% homogenate of virus-infected liver. This viral homogenate was pooled, divided into samples, and stored at −80°C. A sample of the homogenate was thawed, and titers were determined in mice for virus infectivity before use. For in vitro passage, a homogenate of MHV-infected NCTC 1469 mouse liver cells was prepared and similarly stored.

Mice. Female Swiss Webster mice weighing 18 to 23 g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) or from Simonsen Laboratories (Gilroy, Calif.). These mice were designated specific pathogen free and were free of detectable antibodies to MHV. They were fed mouse chow and tap water ad libitum.

Compounds. PR-879-317A was synthesized as the hydrochloride salt by chemists at Pennwalt Corp. (Rochester, N.Y.). Levamisole was obtained from Sigma Chemical Co. (St. Louis, Mo.). Ribavirin was obtained from ICN Pharmaceuticals Inc. (Irvine, Calif.). Both PR-879-317A and levamisole were dissolved in sterile physiological saline for the in vivo animal experiments. For the in vitro experiments, PR-879-317A and ribavirin were dissolved in cell culture medium.

In vitro experiments. Mouse liver cells (NCTC clone 1469 obtained from ATCC) were grown in 96-well microplates (NUNC; Vangard International, Neptune, N.J.) for 18 h before treatment with various concentrations of PR-879-317A. The drug was added to the cultures 15 min before exposure to MHV. Viral cytopathic effect (CPE) was determined microscopically 96 h after virus introduction, and antiviral activity was calculated as a function of the degree of

* Corresponding author.

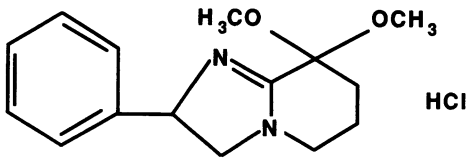

FIG. 1. Structure of 2,3,5,6,7,8-hexahydro-2-phenyl-8,8-dimethoxy-imidazo[1,2-*a*]pyridine (PR-879-317A).

inhibition of CPE (11). Ribavirin at the same concentrations was also used in each experiment as a positive control.

Titration of liver virus. Liver homogenates were prepared in minimum essential medium to a 10% (wt/vol) suspension. These homogenates were diluted through 10^{-5} and added in 0.2-ml portions to the NCTC cell monolayer. Triplicate wells were used for each dilution. Viral CPE was determined 72 h later. Fifty-percent endpoints were determined by the method of Reed and Muench (7).

In vivo experiments. A series of in vivo experiments was conducted to assess various aspects of the antiviral properties of PR-879-317A. Although the specific experimental designs of these studies varied, the general protocols were always consistent. In all studies, the antiviral effects of PR-879-317A or levamisole were assessed in mice which had received an i.p. dose of MHV normally sufficient to ultimately prove lethal in approximately 90% of animals. This mouse-passaged virus dose was equivalent to 0.1 cell culture 50% infectious dose per ml in NCTC 1469 cells. As will be seen in these studies, different lots of mice, although of the same age, sex, and species, responded differently to the same virus inoculum. To evaluate drug effects in the absence of viral infection, the same doses of PR-879-317A or levamisole were also administered to groups of mice which had been i.p. dosed with only the vehicle for the MHV and which therefore remained uninfected.

At 4 to 5 days after administration of the virus (or virus vehicle) one-half of the mice from each treatment group were killed, the livers were removed, and the blood was collected. The resultant serum samples were separated and frozen for subsequent assay of serum glutamic oxaloacetic and pyruvic transaminases (SGOT, SGPT) and, in one experiment, for measurement of bilirubin levels by using colorimetric kits obtained from Sigma. SGPT titers greater than 400 Sigma-Frankel (SF) units/ml or SGOT titers in excess of 900 SF units/ml were considered positive and indicative of liver damage. These positive values were determined on the basis of repeated studies in uninfected mice in which the SGOT and SGPT titers never exceeded the values indicated. Animals that died before the scheduled time of sacrifice were considered to have positive SGOT and SGPT titers. The livers were examined for determination of the extent of hepatic damage on a scale of 0 to 4 (12): 0 = normal liver; 1 = <25% discoloration; 2 = 26 to 50% discoloration; 3 = 51 to 75% discoloration; 4 = >75% discoloration. We have found liver discoloration to coincide well with histopathologic changes in the liver caused by the virus infection and to particularly coincide with rises in SGOT and SGPT. The livers were labeled, photographed, and frozen for later virus assay.

The remaining half of the mice from each treatment group were observed daily for up to 21 days to ascertain survival rates.

In the initial experiment, PR-879-317A was administered i.p. once daily for 3 days beginning 48 h before virus inoculation. Four doses of the compound were used, including 3.2, 6.3, 12.5, and 25 mg/kg per day. The second experiment evaluated the importance of the timing of PR-879-317A treatment in relation to inoculation of the virus. In this experiment, PR-879-317A (12.5 and 25 mg/kg per day) was administered i.p. twice daily for 3 consecutive days. In separate studies, treatment initiation was timed to begin at either 24 or 48 h before virus inoculation or at 16 h after inoculation. Antiviral efficacy was judged as a function of improvements in survivors, mean survival time, and liver damage scores.

A series of experiments assessed the antiviral efficacy of PR-879-317A when administered in various distinct treatment schedules. All multiple-dose regimens included i.p. dosing beginning 48 h before inoculation. These regimens included dosing once, twice, or three times daily for 3 consecutive days, dosing every other day through 3 days, and dosing once daily for 5 consecutive days; all studies incorporated administration of PR-879-317A doses in the range of 0.8 to 25 mg/kg per day. In two additional studies, PR-879-317A was administered i.p. only once at 24 or 48 h before inoculation, at doses of 3.1, 6.3, 12.5, and 25 mg/kg. The percent change in both the 21-day survival frequency and the mean liver scores was calculated for each individual experiment against the appropriate saline-treated group.

Another experiment delineated the relative antiviral efficacy of various dosing routes of PR-879-317A including oral (p.o.), i.p., intramuscular (i.m.), and subcutaneous (s.c.), when given once daily for 3 consecutive days beginning 24 h after virus inoculation. An analogous study incorporated once-daily oral dosing of PR-879-317A for 3 days beginning 24 h before virus inoculation. Drug doses in these studies were in the range of 0.8 to 25 mg/kg per day.

An experiment was done to compare the antiviral effects of equal doses of PR-879-317A or levamisole (6.3, 12.5, and 25 mg/kg per day); both compounds were given i.p. for 3 consecutive days beginning 24 h after virus inoculation.

In all experiments, the uninfected animals were housed in rooms separate from those of infected mice. When drug treatment began after virus inoculation, the mice were randomized after infection but before drug treatment. When drug treatment began before virus infection, all mice at a single dosage level were randomized before virus inoculation.

Statistical analyses. Increases in mean survival time were evaluated by using Student's *t* test, critical to a 5% significance level. Survival rates, frequencies of abnormal SGOT and SGPT serum levels, and hepatic viral titers were analyzed by using chi-square analysis with Yate's correction. The Wilcoxon ranked sum analysis was used for evaluating the mean liver score. Means are expressed with standard deviations.

RESULTS

Two in vitro anti-MHV experiments were done with PR-879-317A and ribavirin. In both experiments, PR-879-317A failed to exhibit an antiviral effect (50% effective dose, >320 μg/ml), whereas ribavirin was significantly inhibitory to the virus, with a 50% effective dose of 3.2 to 10 μg/ml.

Results from the study in which PR-879-317A was administered once daily for 3 days beginning 48 h before virus inoculation are summarized in Table 1. In mice which did not receive PR-879-317A, the MHV infection resulted in the death of 80% of animals; the mean survival time was 6.3 days. When killed 4 days after inoculation, mice which did not receive PR-879-317A had markedly discolored livers

(mean liver score of 2.7) and their liver virus titer was $10^{4.8}$. Likewise, 60% of these infected mice had elevated SGOT and SGPT levels, indicative of hepatic damage. For comparison, all uninfected mice had livers which appeared normal, SGOT and SGPT levels in the normal range, and no detectable hepatic virus titers. All doses of PR-879-317A decreased the extent of liver discoloration and markedly increased the number of mice which survived for 21 days. Mice treated with 6.3 or 25 mg of PR-879-317A per kg also exhibited a lower incidence of positive SGOT and SGPT titers. PR-879-317A also tended to reduce hepatic virus titers, being statistically significant at doses of 3.2 and 6.3 mg/kg per day.

The effect of varying the times of initiation of PR-879-317A therapy on MHV infections was determined, with treatments i.p. twice a day for 3 days begun 48 h before, 24 h before, or 16 h after virus inoculation. In the saline-treated groups, only 15% of the mice survived through 21 days (data not shown). The saline-treated mice sacrificed at day 4 after virus inoculation displayed a severe infection (mean liver score of 3.4). The i.p. administration of PR-879-317A significantly increased the 21-day survival frequency in the 16 h after virus inoculation and the 48-h pre-virus inoculation groups. A significant reduction in the extent of liver discoloration was noted in both the 48- and 24-h preinoculation groups. These data indicate that all three treatment initiation times studied were efficacious.

Several studies were done in which PR-879-317A was administered on different treatment schedules to mice infected with MHV. Dosage schedules studied were once daily for 3 or 5 days, twice daily for 3 days, three times daily for 3 days, and once every other day for 3 days, all initiated 48 h before virus inoculation. In addition, we studied the effects of single treatments 4 h before or 24 h after MHV inoculation. All treatments were i.p., with doses of 6.3, 12.5, and 25 mg/kg per day. The single treatments also used a 3.1-mg/kg dose. Multiple prophylactic dosing with PR-879-317A appeared to be effective when given either once or twice but not three times daily (data not shown). Liver discoloration and survival frequency were significantly improved in the once-daily group receiving 6.3 mg/kg per day when the mice were treated for only 3 days, as well as in the 12.5- and 6.3-mg/kg per day dose groups in the twice-daily regimen. Prolongation of the once-daily dosing regimen for 5 days virtually eliminated the antiviral effects. Drug administration on an alternate-day regimen proved effective only at the highest (25 mg/kg per day) dose level tested, as shown by the

TABLE 1. Effect of i.p. administration of PR-879-317A once daily for 3 consecutive days beginning 48 h before inoculation with MHV

Dose (mg/kg per day)	No. of mice	% 21-day survival	Mean liver score[a]	% SGOT positive[b]	% SGPT positive[c]	Liver virus titer, $\log_{10}$[d]
0	20	20	2.7 ± 0.3	60	60	4.8 ± 0.4
3.2	10	90[e]	1.4 ± 0.6[e]	40	40	3.5 ± 0.3[f]
6.3	10	90[e]	1.1 ± 0.5[e]	20[f]	20[f]	3.0 ± 0.2[f]
12.5	10	80[e]	1.6 ± 0.6[e]	40	40	4.3 ± 0.4
25	10	80[e]	1.2 ± 0.5[e]	30[f]	20[f]	3.2 ± 0.4

[a] Scored from 0 (normal) to 4 (severe discoloration or death).
[b] SGOT levels, >900 SF units/ml.
[c] SGPT levels, >400 SF units/ml.
[d] Reciprocal of dilution of liver homogenate per milliliter causing 50% viral CPE in NCTC 1469 cells.
[e] Significantly different from corresponding 0 dose value. $P < 0.01$.
[f] Significantly different from corresponding 0 dose value. $P < 0.05$.

TABLE 2. Effect of oral administration of PR-879-317A twice daily for 3 consecutive days, beginning 24 h before inoculation with MHV

Dose (mg/kg per day)	No. of mice	% 21-day survival	Mean liver score[a]	No. SGOT positive[b] /total	No. SGPT positive[c] /total	Liver virus titer, $\log_{10}$[d]
0	20	50	2.4 ± 0.4	4/14[e]	11/14	2.7 ± 0.7
0.8	10	80[f]	1.3 ± 0.4[f]	0/8	0/8[g]	0.0[g]
1.6	10	90[f]	0.9 ± 0.4[g]	0/9	0/9[g]	0.0[g]
3.1	10	80[f]	1.4 ± 0.4[f]	0/8	0/8[g]	0.0[g]
6.3	10	90[f]	1.5 ± 0.6[f]	1/8	1/8[g]	0.3 ± 0.3[g]
12.5	10	70	1.4 ± 0.4[f]	0/8	0/8[g]	0.0[g]
25	10	70	1.0 ± 0.4[g]	1/10	1/10[g]	0.6 ± 0.4[g]

[a] Scored from 0 (normal) to 4 (severe discoloration or death).
[b] SGOT levels, >900 SF units/ml.
[c] SGPT levels, >400 SF units/ml.
[d] Reciprocal of dilution of liver homogenate per milliliter causing 50% viral CPE in NCTC 1469 cells.
[e] Totals reduced because of insufficient serum in some assays.
[f] $P < 0.05$.
[g] $P < 0.01$.

significant increase in survival frequency and reduction in liver score. The most effective dosing regimens appeared to be those with the single treatment. In the single-injection prophylactic study, all four doses significantly increased the survival rates, with a concomitant reduction in hepatic discoloration seen in both the highest (25 mg/kg per day) and lowest (3.1 mg/kg per day) dose groups. In the single-dose therapeutic study, animals receiving 3.1, 6.3, or 25 mg of PR-879-317A per kg per day displayed a significant increase in the 21-day survival frequency; the 3.1- and 12.5-mg/kg per day dose groups also displayed improved liver function, as judged by hepatic discoloration (data not shown).

A study was done comparing the effect of p.o., i.p., i.m., and s.c. administration of PR-879-317A on its antiviral activity in the MHV system. Doses of 6.3, 12.5, and 25 mg/kg per day were used. The compound was administered once daily for 3 days beginning 24 h after virus inoculation. The i.p. administration of PR-879-317A appeared to be the most effective under these dosing conditions, with significant increases in survival frequency and reduced liver scores at all dosage levels (data not shown). The groups given three doses of PR-879-317A p.o. all demonstrated significant reductions in hepatic discoloration, but no significant increase in survival frequency was seen. The s.c. and i.m. routes of administration were ineffective in this test model; no significant changes in either hepatic discoloration or the 21-day survival frequency was noted at any of the dose levels.

Table 2 describes results from an oral dosing study in which PR-879-317A was administered twice daily for 3 days starting 24 h before MHV inoculation. In this case, mice inoculated with the virus and treated with a placebo experienced only a 50% survival rate through 21 days. The placebo-treated mice killed at 5 days after inoculation showed signs of only a moderately severe infection, suggestive of a weaker virus inoculum in this experiment. These mice had mean liver scores of 2.4 and hepatic viral titers of $10^{2.7}$; the incidence of elevated SGOT and SGPT levels was relatively low. In the face of this lower oral challenge, marked activity was seen at all dose levels by using all evaluation parameters.

Results of the comparative antiviral effects of PR-879-317A and levamisole are presented in Table 3. In mice which received a placebo only, the MHV infection killed 95% of animals tested and their mean survival time was 5.4 days. At

TABLE 3. Effect of i.p. administration of PR-879-317A or levamisole, once daily for 3 consecutive days, beginning 24 h after inoculation with MHV

Compound	Dose (mg/kg per day)	No. of mice	% 21-day survival	Mean liver score[a]	Serum bilirubin (mg/100 ml)	Mean virus titer, $\log_{10}$[b]
Saline	0	20	5	2.6 ± 0.3	0.9 ± 0.4	3.5 ± 0.2
PR-879-317A	6.3	10	40[c]	1.7 ± 0.5	0.5 ± 0.1	2.3 ± 0.1
	12.5	10	70[c]	1.0 ± 0.5[c]	0.5 ± 0.2	0.2 ± 0.2[c]
	25	10	50[c]	1.3 ± 0.6[c]	0.4 ± 0.2	0.3 ± 0.1[c]
Levamisole	6.3	10	60[c]	1.8 ± 0.6	0.9 ± 0.2	2.3 ± 0.3
	12.5	10	80[c]	2.3 ± 0.5	0.6 ± 0.2	2.5 ± 0.2
	25	10	10	3.1 ± 0.4	0.2 ± 0.2	3.5 ± 0.5

[a] Scored from 0 (normal) to 4 (severe discoloration or death).
[b] Reciprocal of dilution of liver homogenate per milliliter causing 50% viral CPE in NCTC 1469 cells.
[c] $P < 0.01$.

4 days after inoculation the mean liver score was 2.6, the serum bilirubin level of infected mice was 0.9, and the corresponding liver virus titer was $10^{3.5}$. All doses of PR-879-317A markedly increased the number of mice which survived for 21 days and consistently tended to reduce serum bilirubin toward normal levels. The doses of 12.5 and 25 mg/kg per day of PR-879-317A also significantly reduced the degree of observable liver damage and dramatically reduced hepatic virus titers. For comparison, in this specific dosing regimen, the highest dose of levamisole did not increase survival frequency of infected mice, although the lower doses were effective. Levamisole also did not prevent virus-induced hepatic discoloration or reduce hepatic virus titers. The serum bilirubin values were determined only in this experiment and were not found to react as strongly to the MHV infection as did SGOT and SGPT. This experiment was run relatively early in our MHV studies; for the later studies, serum transaminase levels were evaluated instead of bilirubin because of the more striking SGOT and SGPT responses to the infection.

Toxicity controls were run in parallel with every experiment. In no case did any of these animals die during the 21-day duration of the study, although slightly reduced weight gain during the period of treatment was observed in animals receiving the 25-mg/kg per day treatments. Preliminary dose range-finding studies revealed that the 10% lethal dose of PR-879-317A was approximately 50 mg/kg per day when given i.p. during 3 days.

DISCUSSION

The immunorestorative agent PR-879-317A has now been shown to have significant antiviral activity when administered to mice infected with MHV. The activity seen is reproducible, is achieved when the compound is given either p.o. or i.p., and occurs when treatment is initiated either before or after virus inoculation. Antiviral activity was exhibited through a spectrum of dosage levels which were well tolerated by the mice.

The MHV was selected as a target virus for these studies because the disease it induces is a reasonable model for type A (infectious) hepatitis (10) induced by infectious hepatitis virus. The only animals susceptible to infectious hepatitis virus are chimpanzees and marmoset monkeys, which are both in short supply and very expensive. Like the infectious hepatitis virus, the MHV is an RNA-containing agent, and MHV induces an infection in mice which resembles the hepatic disease induced by infectious hepatitis virus. We have used the MHV infection in previous chemotherapy studies and found ribavirin to have a marked effect in this model (12). Ribavirin has been reported to be effective against type A hepatitis in humans (1, 13), suggesting that activity against MHV infections may be predictive of activity against the corresponding human disease.

In these studies, the anti-MHV activity induced by PR-879-317A appeared to exceed that induced by levamisole, an immunomodulatory substance (8, 14). Levamisole administered in drinking water has been reported to have a slight effect in increasing survival time in animals infected with MHV (15). The s.c. administration of levamisole also was noted to be effective in rats infected with herpes simplex type 2 virus (2, 3). Improvement of disease signs was seen when a single i.m. injection of levamisole was given to cattle or goats infected with foot-and-mouth disease virus (9). Incorporation of the compound into the diet of mink infected with Aleutian disease virus also appeared to improve their health (4). These reports indicating that levamisole possesses antiviral activity in certain situations suggest other potential uses for PR-879-317A.

Although the MHV used in these studies consistently had the same titer in susceptible NCTC 1469 mouse liver cells, we found that the titer varied considerably among lots of mice. We cannot explain this variation among lots of mice; the differences were not dependent upon the supplier, and all the animals were certified by the suppliers to be free of MHV antibodies. Repeated titrations of the virus in separate lots of mice resulted in consistent infectivity rates within those lots. Although death rates and liver virus titers varied appreciably among lots of mice, the liver scores were relatively consistent throughout, indicating that liver damage did not always correlate with recoverable virus titer or actual death of the animal. We have no doubt that the occasional lesser infection seen may have enhanced the antiviral activity expressed in a particular experiment.

PR-879-317A appears to be a humoral and cellular immunorestorative agent. In immunodeficient mice, the compound augmented antibody production to sheep erythrocytes and enhanced blastogenic responses of normal murine splenocytes to phytohemagglutinin and concanavalin A (Radov et al., in press). After incubation of peripheral blood mononuclear cells obtained from Down's syndrome patients, PR-879-317A increased activity of natural killer cell function, antibody-dependent cell-mediated cytotoxic activity, and mitogenic responses to phytohemagglutinin, concanavalin A, and pokeweed mitogen and stimulated production of migration inhibition factor (Warren et al., in press). In mice infected with MHV and treated with PR-879-317A, increased splenic production of interleukin-1, interleukin-2, and antibody-dependent cell-mediated cytotoxicity was observed (manuscript in preparation). This compound is

well absorbed when administered p.o. or i.p. to mice, with serum level half-lives of approximately 1 h (unpublished data, Pennwalt Corp.). A definite T-cell effect is seen when the compound is administered by either route. No data are available on absorption after i.m. or s.c. injection of the material; the data from this MHV study suggest that these latter routes are ineffective, perhaps because of absorption differences.

The lack of activity in our in vitro antiviral assay strengthens the premise that the in vivo antiviral activity exhibited by PR-879-317A results from immune modulation rather than a direct antiviral effect. The immunorestorative activity of PR-879-317A might also explain why excessive administration of the drug (twice daily, or twice daily versus once daily in some studies, and 5 days versus 3 days of therapy) could paradoxically reduce its antiviral activity, whereas such overstimulation of the immune system of the host could activate compensatory mechanisms to depress immune responsiveness.

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