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Treatment of Coxsackievirus A9 Myocarditis in Mice with WIN 54954

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The therapeutic efficacy of an experimental antiviral agent, WIN 54954, was evaluated in murine myocardial infection with coxsackievirus A9 (CVA9). Eight-month-old male Swiss Webster mice were inoculated with 1.5 × 10^4 PFU of CVA9, Boston strain 13. WIN 54954, a broad-spectrum antipicornavirus agent, was administered orally in a dose of 0.25, 2.5, 25, 50, 100, or 200 mg/kg of body weight per day on days 1 to 3 after virus inoculation. Control animals received xanthan gum carrier only. Mice were sacrificed on day 4. Myocardial titers of virus were determined and found to be significantly lower in the four highest dose treatment groups ($P < 0.001$ for all groups) compared with controls. Heart weights were also significantly lower compared with controls in these four groups ($P < 0.001$ for all groups). When mice received 50 mg of WIN 54954 per kg daily beginning at either 48 or 72 h postinoculation, myocardial titers were once again significantly reduced compared with those of controls ($P < 0.001$ for both groups). Neurological toxicity was observed in the 100- and 200-mg/kg/day groups but not in the lower-dose groups. Thus, WIN 54954 effectively reduced myocardial CVA9 replication in a murine model.

Viruses are believed to be the etiologic agents of most of the clinically detected cases of myocarditis in the United States (13). A wide spectrum of disease has been described, ranging from transient inflammation to a fulminant course which can culminate in heart failure or sudden death (1, 6, 16). The most frequently detected etiologic organisms are the enteroviruses, especially the coxsackieviruses (9). Unfortunately, treatment is now largely supportive because of a variety of factors, including a poor understanding of the pathogenesis of the disease and a lack of effective antiviral agents.

Although mechanisms of viral myocarditis have been difficult to study in humans, significant progress has been made in experimental murine studies and two important models have been established. In the model of myocarditis produced by coxsackievirus B3 (4, 12), a biphasic disease process has been demonstrated. Initially, the virus achieves high myocardial titers but generates little cytopathic damage. Subsequently, when live virus can no longer be retrieved from the heart, a severe T-cell-mediated inflammatory process supervenes, characterized by diffuse myocardial inflammation and necrosis (11).

The second model uses infection with coxsackievirus A9 (CVA9), which causes direct destruction of myocardial cells (8, 15). Initially, the infection produces a significant viremia, when little virus can be isolated from the heart. Later, as titers fall in the blood, they rise in the myocardium. The virus causes myocarditis primarily in adult mice, producing a focal inflammation and achieving high titers in affected myocardia. The disease is more severe in male mice and when accompanied by immune suppression or exercise (14).

The WIN class of drugs consists of synthetic antiviral agents that bind to the protein shell or capsid of many picornaviruses and inhibit viral attachment and/or uncoating (3, 10). The potency of these drugs has been established in vitro against a broad spectrum of picornaviruses, including most coxsackieviruses, and the efficacy of oral WIN 54954 has been shown in systemic enteroviral infections of suckling mice (17).

In the present study, the efficacy of the antiviral agent WIN 54954 on murine CVA9 myocardial infection has been investigated. A clear antiviral effect has been demonstrated at subtoxic doses.

MATERIALS AND METHODS

Virus. CVA9 Boston strain 13 was prepared in a monolayer of monkey kidney (MK) cells after inoculation with a multiplicity of infection of 10 PFU per cell in Leibovitz's L15 medium supplemented with 420 μg of glutamine, 420 μg of arginine, 400 U of penicillin, 40 μg of streptomycin, and 400 μg of dextrose, each per milliliter. After adsorption of the virus for 60 min, the medium was further supplemented with fetal bovine serum (FBS) to a final concentration of 10%. The virus was harvested after 24 h, aliquoted, and frozen at −80°C until ready for use.

Virus titers were determined on monolayers of human foreskin fibroblasts by using the plaque technique. Decimal dilutions of either the virus stock or a virus-containing tissue were inoculated in 0.5-ml volumes of supplemented L15 medium without FBS onto human foreskin fibroblast monolayers on 35-mm Corning cell culture plates. After incubation for 60 min at 37°C, the monolayers were washed once with 1.0 ml of L15 diluent and overlaid with 3.0 ml of clear L15 agar containing 10% FBS. After 24 and 120 h of incubation at 37°C, the cells were treated with an additional 1.0 ml of agar with and without, respectively, 120 μg of the vital stain neutral red per ml. The monolayers were observed daily, and the maximal plaque counts were recorded by day 7.

Mice. Eight-month-old male Swiss Webster mice were obtained from Simonsen Farms, Gilroy, Calif.

Antiviral drug. WIN 54954 was obtained from Sterling-Winthrop Research Institute, Renssalaer, N.Y. For in vitro studies, WIN 54954 was dissolved in dimethyl sulfoxide and

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diluted in supplemented L15 medium without FBS. For in vivo studies in mice, WIN 54954 was suspended daily in 0.5% xanthan gum.

In vitro activity and toxicity. Virus activity was determined in MK cell monolayers in the presence or absence of serial threefold dilutions of WIN 54954. The concentration of WIN 54954 that resulted in a 50% reduction in the number of PFU as compared to that measured simultaneously in the control lacking drug was determined from a plot of percent reduction of PFU versus concentration. Cellular toxicity was assessed by recording morphology and monolayer formation of MK cells grown in the presence of threefold-increasing concentrations of WIN 54954.

Experimental design. To examine the effect of drug dose, mice were inoculated intraperitoneally with 1.5×10^4 PFU of virus in 0.05 ml of L15 diluent. At 24 h after inoculation, a twice-daily regimen of either 0.2 ml of placebo (0.5% xanthan gum) or 0.2 ml of either of two different concentrations of WIN 54954 in xanthan gum was initiated. The respective treatment was administered intragastrically with a gavage needle and continued for a total of 3 days. Sequential experiments with each of three pairs of drug concentrations, 100 and 200 mg/kg of body weight per day, 25 and 50 mg/kg/day, or 0.25 and 2.5 mg/kg/day, were performed.

To determine the effect of delayed initiation of treatment, mice were inoculated intraperitoneally with 1.5×10^4 PFU of virus in 0.5 ml. Test mice received WIN 54954 intragastrically at 50 mg/kg/day twice a day beginning at either 48 or 72 h postinoculation. Control mice received xanthan gum only.

For both protocols, all mice were observed daily and clinical findings were noted. Complete necropsies were performed either on day 4, when mice were sacrificed by inhalation of methoxyflurane, or earlier, in the event of premature death.

Tissue processing and virus isolation. Hearts and spleens were weighed aseptically and homogenized as a 1:6 solution in L15 medium supplemented with 50% FBS. After centrifugation at $1,000 \times g$ for 15 min, dilutions of the supernatants were inoculated in 0.5-ml volumes onto human foreskin fibroblast monolayers. Virus titers were determined by plaque assay as noted above. A portion of brain tissue was processed as described above in a 1:8 suspension and assayed for virus. Aliquots (0.3 ml) of blood were homogenized as a 1:6 solution in L15 medium supplemented with 50% FBS, and virus titers were determined by plaque assay.

A portion of brain tissue was fixed in 37% formalin and stained with hematoxylin and eosin. The tissue was then examined by light microscopy for evidence of cellular necrosis and inflammation.

Statistics. Experimental means were calculated and then compared by the standard two-tailed Student's *t* test.

RESULTS

In vitro studies. The 50% plaque inhibitory concentration of WIN 54954 for CVA9 Boston strain 13 was 0.0003 μg/ml in MK cells. In rapidly dividing MK cells, the 50% cell growth inhibitory concentration was 10 μg/ml, and 30 μg/ml was associated with rounding of cells and gross monolayer degeneration.

In vivo tolerance. All mice in the control groups survived through day 4 and failed to manifest any abnormal clinical findings (Table 1). Two animals, one in the 25-mg/kg/day treatment group of the experiment evaluating the effect of drug dose and one in the 48-h treatment group of the experiment evaluating delayed initiation of treatment (50 mg/kg/day), died prematurely because of esophageal rupture. The trauma was presumably sustained in both cases during one of the feedings. No other mouse in the 0.25-, 2.5-, 25-, or 50-mg/kg/day treatment groups died prematurely, and no abnormal clinical findings were observed. However, of the mice treated with a dose of 100 mg/kg/day, one died prematurely of a seizure and one other mouse demonstrated ataxia. In the 200-mg/kg/day treatment group, five mice were ataxic and three of these died prematurely. No additional abnormal findings were noted in these two groups.

TABLE 1. Clinical findings in mice inoculated wtih CVA9

Treatment group and dose (mg/kg)	Total no. of mice	No. of mice with abnormal clinical findings		
		Seizure	Ataxia	Premature death
Control	30	0	0	0
Treated with WIN 54954				
0.25	10	0	0	0
2.5	10	0	0	0
25	10	0	0	1[a]
50	10	0	0	0
100	10	1	1	1
200	10	0	5	3

[a] Esophageal rupture noted at autopsy.

To assess the possible causes for the clinical central nervous system abnormalities observed, the brains of all mice treated with at least 100 mg/kg/day were examined for cellular necrosis and inflammatory changes and the titers of the virus were determined. No significant differences were found in the titers of the virus in the brains of the 13 treated mice who demonstrated abnormal clinical findings and/or experienced premature death [mean ± standard deviation, $(1.5 \pm 1.3) \times 10^2$ PFU/ml] compared to the 7 asymptomatic, treated mice [$(1.2 \pm 0.8) \times 10^2$ PFU/ml]. Furthermore, no pathologic changes were found in any of the mice.

In vivo antiviral effects. Myocardial titers of virus were significantly lower in the mice of the four highest dose groups compared to those of respective controls ($P < 0.001$ for all groups; Table 2). No significant differences existed among these four treatment groups. Despite the low titers of virus recovered from the blood and spleens of control mice, significantly less virus was generally isolated from the blood and spleens of the four highest dose treatment groups than from those of the respective controls ($P < 0.02$; Table 2). Once again, there was no significant different between these four treatment groups.

The effect of delayed onset of treatment was determined by initiating treatment at either 48 or 72 h after inoculation of virus. The concentrations of virus in the hearts were again reduced at 48 h [mean ± standard deviation, $(8.7 \pm 3.1) \times 10^2$ PFU/ml] or 72 h [$(12.2 \pm 2.6) \times 10^2$ PFU/ml] when compared to the controls [$(20.5 \pm 4.7) \times 10^2$ PFU/ml; $P < 0.001$].

In vivo therapeutic effect. Heart weights were significantly lower in the four highest dose groups than those of the controls ($P < .001$ for all groups; Table 3). No significant difference existed among these four treatment groups.

TABLE 2. Effect of WIN 54954 on CVA9 titers in the heart, blood, and spleen of mice treated on days 1 through 3

Titer measured in:	WIN 54954 dose (mg/kg)	No. of mice	CVA9 titer (PFU/ml) (10^2)		P (vs. controls)
			Mean ± SD	Range	
Heart	None (control)	10	38.8 ± 8.7	24–54	
	0.25	10	34.3 ± 7.1	28–46	NS[a]
	2.5	10	38.3 ± 8.1	28–48	NS
	None (control)	10	35.7 ± 16.1	6–66	
	25	9	5.7 ± 3.5	2–12	<0.001
	50	10	5.2 ± 3.8	1–12	<0.001
	None (control)	10	56.1 ± 19.9	34–107	
	100	9	8.2 ± 3.9	3–15	<0.001
	200	7	3.6 ± 1.5	0–6	<0.001
Blood	None (control)	10	6.6 ± 3.1	4–12	
	25	9	2.5 ± 2.0	0–6	<0.01
	50	10	1.8 ± 1.5	0–8	<0.001
	None (control)	10	2.4 ± 2.0	0–8	
	100	9	0.7 ± 0.5	0–2	<0.02
	200	7	0.2 ± 0.1	0–1	<0.01
Spleen	None (control)	10	3.1 ± 1.9	0–7	
	25	9	0.5 ± 0.4	0–2	<0.001
	50	10	1.8 ± 1.4	0–4	NS
	None (control)	10	8.0 ± 3.4	3–12	
	100	9	3.0 ± 1.7	0–8	<0.001
	200	7	3.4 ± 1.3	2–6	<0.01

[a] NS, not significant.

DISCUSSION

The enteroviruses are responsible for a variety of clinical manifestations including exanthems, herpangina, myositis, pleuritis, aseptic meningitis, and myocarditis. Unfortunately, as of the current date, no antiviral agent has been demonstrated to be sufficiently safe and effective versus enteroviruses to be approved for clinical use by the Food and Drug Administration.

Members of the WIN class of synthetic antiviral drugs have been shown to be effective against many enteroviruses both in vitro and in vivo. Thus, in the present study, WIN 54954 was found to give a 50% reduction in the number of PFU of CVA9 at a concentration of 0.0003 μg/ml. Pharmacokinetic studies by Woods et al. have demonstrated maximal serum levels of 0.12 to 3.41 μg/ml in suckling mice administered WIN 54954 in doses ranging from 2 to 100 mg/kg/day, respectively (17). In murine efficacy studies, Jubelt et al. have shown with a mouse model of enterovirus encephalitis that WIN 51711, another member of the WIN class, is effective in vivo if initiated intragastrically as late as 20 days after inoculation of virus (5).

TABLE 3. Effect of WIN 54954 on heart weights

WIN 54954 dose (mg/kg)	No. of mice	Heart weight (mg)		P (vs. controls)
		Mean ± SD	Range	
None (control)	10	202 ± 19.6	184–244	
0.25	10	208 ± 19.4	168–226	NS[a]
2.5	10	192 ± 24.7	145–232	NS
None (control)	10	204 ± 20.7	180–238	
25	9	148 ± 14.8	132–167	<0.001
50	10	162 ± 17.9	144–184	<0.001
None (control)	10	216 ± 18.5	201–246	
100	9	149 ± 13.3	136–169	<0.001
200	7	164 ± 17.2	138–180	<0.001

[a] NS, not significant.

Since myocarditis is one of the most challenging syndromes caused by enteroviruses in humans, it was decided to test a WIN compound by using the CVA9 model of myocarditis in adult mice. It is known that after intraperitoneal inoculation of CVA9, the virus titer in the blood rises quickly, peaks on day 2, and subsequently falls (7). In contrast, the virus titer in the myocardium rises slowly and peaks on day 4. This phenomenon was supported by data from the placebo-fed controls of the present study, since at sacrifice on day 4 the titer of the virus was significantly greater in the hearts than in either the blood or the spleens. Of greater interest in the present study, a concentration of intragastrically administered WIN 54954 as low as 25 mg/kg/day significantly reduced the titer of virus in the myocardia, blood, and spleens of the respective placebo-treated controls. In fact, if the administration of the antiviral agent was initiated as late as 72 h after inoculation of CVA9, there was still a significant reduction in the titer of the virus in the myocardia of test mice.

Earlier work had shown that adult mice infected with the same strain of CVA9 had significantly greater heart weights than did uninfected controls (2). In the present study, those concentrations of WIN 54954 associated with a significantly lower titer of virus in the myocardium were also associated with a significantly lower heart weight. Thus, there was a likely clinical correlate to the antiviral effect demonstrated by WIN 54954. A histologic evaluation of the myocardium was not carried out since preliminary studies confirmed the earlier observations that although virus titers are highest on day 4, there are not yet pathologic changes (7).

A previous study by Woods et al. failed to demonstrate toxicity of WIN 54954 when administered to mice in doses up to 100 mg/kg/day (17). In the present study, neurologic toxicity was manifested by WIN 54954 in the mice if the drug was administered at a dose of 100 mg/kg/day or greater. Since the therapeutic effect could be demonstrated with a dose as low as 25 mg/kg/day, there was a range of doses that were effective but not toxic in the mouse.

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