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Evaluation of Various Analogues of Tilorone Hydrochloride Against Venezuelan Equine Encephalitis Virus in Mice

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The antiviral activity of tilorone hydrochloride and three of its analogues (11,002, 11,567, and 11,877) was assessed by oral and intraperitoneal (i.p.) administration to Venezuelan equine encephalitis (VEE) virus-infected mice. Significant increases in the percentage of survival ($P < 0.01$) were apparent after oral administration of tilorone and analogue 11,877 at dosages of 250 and 500 mg/kg. Neither tilorone nor 11,877 increased percentage of survival when dosages of 31.25 to 500 mg/kg were given by the i.p. route. Orally administered analogue 11,002 was effective against 100 mouse intracranial median lethal doses ($MICLD_{50}$) of VEE virus at doses at 250 to 1,000 mg/kg; doses of 31.25 to 250 mg/kg given i.p. were effective against 10 $MICLD_{50}$. Oral dosages of 250 to 1,000 mg of analogue 11,567 per kg were active against 100 $MICLD_{50}$ of virus. By the i.p. route, 250 mg of 11,567 per kg protected mice against 1,000 $MICLD_{50}$, and a dose of 125 mg/kg protected against 100 $MICLD_{50}$. Oral treatment of VEE infection with analogue 11,567 24 h after subcutaneous inoculation of VEE virus resulted in no significant increase in the percentage of survivors. All survivors of these studies were susceptible to rechallenge 21 days after the first inoculation of virus.

Numerous substances induce interferon both in vitro and in vivo. Interferon inducers appear to be promising antiviral compounds for protection of humans and animals from viral infections (4, 5, 12). Tilorone hydrochloride, the water-soluble dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]fluorene-9-one, is a low-molecular-weight, broad-spectrum, antiviral agent that presumably acts by inducing endogenous interferon (1, 10). In mice, prophylactic oral administration of tilorone hydrochloride has been shown to be effective against vesicular stomatitis virus (VSV), mengovirus, encephalomyocarditis virus, influenza virus, and herpes simplex virus (8). Intraperitoneal (i.p.) administration of tilorone is protective against the virus designated MM (3), and it is effective against Semliki forest virus when given by the oral, subcutaneous (s.c.), and i.p. routes (9). Consequently, we have studied protection against Venezuelan equine encephalitis (VEE) virus in mice elicited by different prophylactic dosages of tilorone hydrochloride and three of its analogues given by either the oral or i.p. routes. In addition, results are presented of a preliminary therapeutic study with analogue 11,567 given by the oral route.

MATERIALS AND METHODS

Compounds. Tilorone {2,7-bis[2-(diethylamino)-ethoxy]fluorene-9-one dihydrochloride}, analogue 11,002 {2,7-bis[2-(dimethylamino)acetyl]fluorene dihydrochloride}, analogue 11,567 {2,8-bis[2-(dimethylamino)acetyl]-dibenzofuran dihydrochloride}, and analogue 11,877 {2,8-bis[2-(dimethylamino)acetyl]-dibenzothiophene dihydrochloride} were supplied by Merrell-National Laboratories, Cincinnati, Ohio. All compounds were dissolved in pyrogen-free 0.85% NaCl solution prior to use. Dosages ranged from 31.25 to 250 mg/kg by the i.p. route and from 250 to 1,000 mg/kg by the oral route.

Animals. Weanling outbred albino Swiss male mice (CD-1), weighing 14 to 20 g, were obtained from Charles River Mouse Farms, Inc., Wilmington, Mass. The mice were housed 4 to 6 per pan, fed, and given water ad libitum.

Virus. The Trinidad donkey strain of VEE virus was used in these studies (14). The virus was originally isolated in guinea pigs and has been maintained by 13 serial passages in embryonated chicken eggs. This strain of VEE virus produces fatal paralytic disease in mice inoculated s.c. or i.p. Inocula were diluted in modified Hanks balanced salt solution containing 2% fetal calf serum.

Animal techniques. Virus inoculations administered s.c., containing 0, 10, 100 and 1,000 mouse

intracranial median lethal doses ($MICLD_{50}$), were administered midventrally. Tilorone and the designated analogues were given by gavage or i.p. on a milligram/kilogram of body weight basis. In the prophylaxis experiments, compounds were given 20 to 24 h prior to inoculation of the virus. Group sizes averaged 10 mice, with a total of 200 to 300 mice receiving each compound by the i.p. route and 80 to 120 receiving the compounds by the oral route. Each study included both infected, untreated mice and uninfected, treated mice as controls. Mice were observed daily for 14 days postchallenge. Increased percentage of survivors 14 days after virus inoculation was the criterion selected for evaluation of antiviral activity. Mean death times were calculated only on animals that died during the 14-day observation period and were thus unaffected by survivors. The experimental design of the oral studies was similar to that used in the i.p. studies, except for the dosages of compounds administered. All survivors were rechallenged at 21 days after first challenge with 1,000 $MICLD_{50}$ of Trinidad VEE virus by the s.c. route.

Statistical analysis of data. Independent comparisons were made for each virus dose by partitioning the overall chi-square for the given virus dose into independent chi-squares with single degrees of freedom (16). For a given comparison among virus dose levels, the proportion surviving a zero dose of drug was compared to the response for the lowest given dose of drug. If no significance was found ($P < 0.01$), the two responses were averaged and compared with the response to the next higher dose. This averaging of responses for comparison to the next higher dose was continued until a significant difference was found in the proportion surviving. Data from replicate experiments for each compound did not differ significantly and were combined. Analysis of variance was used to determine significant differences in mean death time.

RESULTS

The effects of i.p. administration of tilorone hydrochloride or one of its three analogues 24 h prior to s.c. inoculation of mice with Trinidad VEE virus are presented in Table 1. Dosages equal to or greater than 125 mg of tilorone hydrochloride per kg, 250 mg of 11,877 per kg, and 500 mg of 11,002 or 11,567 per kg administered by the i.p. route showed significant ($P < 0.01$) toxicity to mice and are not included. Dosages lower than 125 mg of tilorone hydrochloride per kg or 250 mg of 11,877 per kg did not significantly increase the percentage of survival when compared with survival of untreated infected mice. Dosages of 31.25 to 250 mg of 11,002 per kg administered prophylactically protected mice against 10 $MICLD_{50}$ of

TABLE 1. *Effect of prophylactic i.p. administration of tilorone hydrochloride, 11,002, 11,567, or 11,877 on the survival of mice inoculated s.c. with VEE virus*

Dose (mg/kg)	% Survival (survivors/total) at 14 days postchallenge			
	0[a]	10	100	1,000
Tilorone				
0	95 (19/20)	20 (4/20)	0 (0/20)	0 (0/20)
31.25	80 (16/20)	40 (8/20)	10 (2/20)	0 (0/20)
62.5	80 (16/20)	45 (9/20)	15 (3/20)	0 (0/20)
11,002				
0	88 (14/16)	13 (2/16)	0 (0/16)	0 (0/16)
31.25	100 (6/6)	100[b] (6/6)	17 (1/6)	0 (0/6)
62.5	83 (5/6)	50[b] (3/6)	33 (2/6)	0 (0/6)
125	94 (15/16)	56[b] (9/16)	38 (6/16)	0 (0/16)
250	100 (16/16)	69[b] (11/16)	19 (3/16)	0 (0/16)
11,567				
0	88 (14/16)	81 (13/16)	0 (0/16)	0 (0/16)
31.25	90 (9/10)	40 (4/10)	10 (1/10)	0 (0/10)
62.5	80 (8/10)	80 (8/10)	10 (2/20)	0 (0/10)
125	100 (16/16)	100 (16/16)	50[b] (8/16)	6 (1/16)
250	94 (15/16)	88 (14/16)	88[b] (14/16)	31[b] (5/16)
11,877				
0	100 (16/16)	38 (6/16)	0 (0/16)	0 (0/16)
31.25	100 (10/10)	60 (6/10)	0 (0/10)	0 (0/10)
62.5	70 (7/10)	30 (3/10)	20 (2/10)	0 (0/10)
125	81 (13/16)	44 (7/16)	25 (4/16)	13 (2/16)

[a] $MICLD_{50}$ of the virus.

[b] $P < 0.01$ compared to untreated, infected mice.

VEE virus but not against 100 or 1,000 $MICLD_{50}$. Significant antiviral activity was found with 125 mg of 11,567 per kg against 100 $MICLD_{50}$ of virus, and a dose of 250 mg/kg was active against 100 and 1,000 $MICLD_{50}$ of virus.

Typical dose-response curves using analogue 11,877 administered by the i.p. route are shown in Fig. 1. Although no significant increase in percentage survival occurred with any of the i.p. dosages of 11,877 (Table 1), a significant ($P < 0.01$) increase in survival time occurred with a dose of 125 mg/kg against a virus dose of 100 $MICLD_{50}$.

The effects of oral administration of the four compounds on the percentage of survival of mice inoculated s.c. with Trinidad VEE virus are given in Table 2. Preliminary trials indicated that all four compounds were less toxic

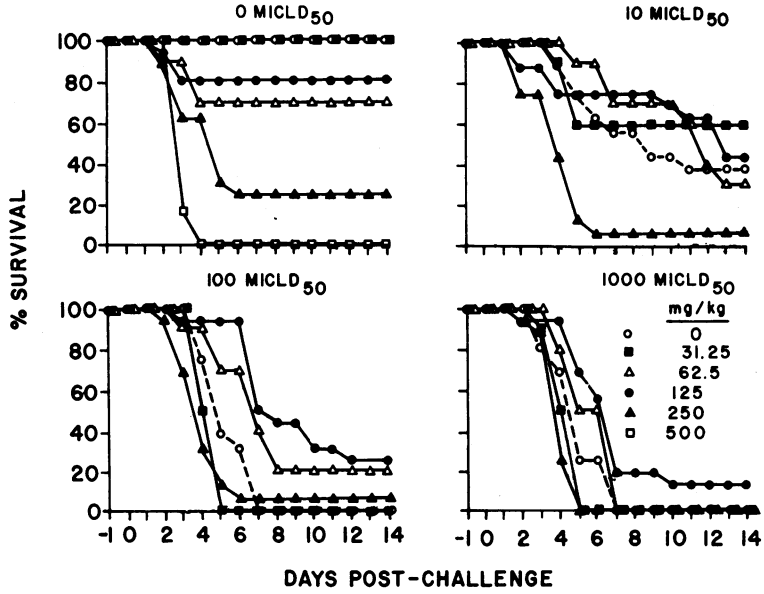

FIG. 1. *Effect of a single i.p. 24-h prophylactic dose of analogue 11,877 on VEE virus infections in mice.*

TABLE 2. *Effect of prophylactic oral administration of tilorone hydrochloride, 11,002, 11,567, or 11,877 on the survival of mice inoculated s.c. with VEE virus*

Dose (mg/kg)	% Survival (survivors/total) at 14 days postchallenge			
	0[a]	10	100	1,000
None	100 (10/10)	20 (2/10)	0 (0/10)	0 (0/10)
Tilorone				
250	90 (9/10)	100[b] (10/10)	70[b] (7/10)	30 (3/10)
500	90 (9/10)	100[b] (10/10)	80[b] (8/10)	23 (2/10)
11,002				
250	100 (10/10)	60[b] (6/10)	50[b] (5/10)	0 (0/10)
500	100 (10/10)	80[b] (8/10)	80[b] (8/10)	30 (3/10)
1,000	100 (10/10)	80[b] (8/10)	90[b] (9/10)	20 (2/10)
11,567				
250	100 (10/10)	80[b] (8/10)	70[b] (7/10)	20 (2/10)
500	100 (10/10)	100[b] (10/10)	60[b] (6/10)	20 (2/10)
1,000	100 (10/10)	100[b] (10/10)	60[b] (6/10)	30 (3/10)
11,877				
250	100 (10/10)	100[b] (10/10)	80[b] (8/10)	10 (1/10)
500	100 (10/10)	100[b] (10/10)	90[b] (9/10)	20 (2/10)
1,000	100 (10/10)	100[b] (10/10)	70[b] (7/10)	50[b] (5/10)

[a] $MICLD_{50}$ of the virus.

[b] $P < 0.01$ compared to untreated, infected mice.

orally than i.p., so higher oral doses could be administered. Only tilorone hydrochloride showed significant toxicity by the oral route at a concentration of 1,000 mg/kg. Lower dosages of tilorone (250 and 500 mg/kg) protected mice against 10 and 100 $MICLD_{50}$ of virus. Oral administration of 250 to 1,000 mg of 11,002, 11,567 or 11,877 per kg protected mice against 10 and 100 $MICLD_{50}$. In addition, 1,000 mg of analogue 11,877 per kg significantly increased the percentage of survival of mice inoculated with 1,000 $MICLD_{50}$ of virus.

Figure 2 depicts typical dose-response curves after oral administration of analogue 11,877 and prior to infection with VEE virus. No significant increases in mean death time were observed in treated mice.

A therapy study was conducted to assess the effect of treatment of VEE virus infection of mice with orally administered analogue 11,567. Groups of mice received 250 or 500 mg of 11,567 per kg 24 h after s.c. inoculation of 0, 10, 100 or 1,000 $MICLD_{50}$ of Trinidad VEE virus. Additional mice were treated with either 250 or 500 mg of 11,567 per kg/day for 3 consecutive days after virus inoculation (Table 3). Neither oral treatment schedule resulted in significant protection against 10, 100 or 1,000 $MICLD_{50}$ of VEE virus. Significant increases in mean death time did not occur as a result of treatment with 11,567 (Fig. 3).

Survivors of all studies were uniformly susceptible to rechallenge with 1,000 $MICLD_{50}$ of Trinidad VEE virus by the s.c. route 21 days after the first administration of virus.

DISCUSSION

Tilorone hydrochloride and three analogues designated 11,002, 11,567 and 11,877 are effective antiviral compounds when given prophy-

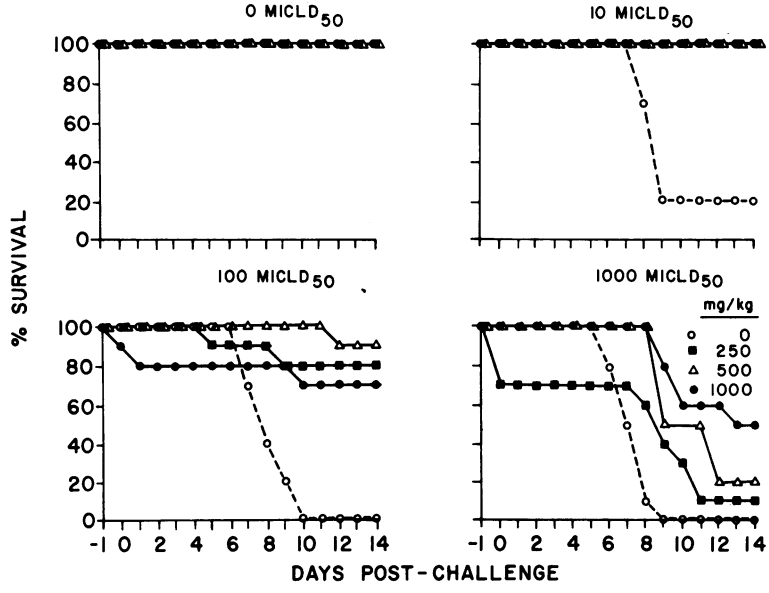

FIG. 2. *Effect of a single oral 24-h prophylactic dose of analogue 11,877 on VEE virus infections in mice.*

TABLE 3. *Effect of therapeutic oral administration of analogue 11,567 on the survival of mice inoculated s.c. with VEE virus*

Dose (mg/kg)	% Survival (survivors/total) at 14 days postchallenge			
	0[a]	10	100	1,000
24 h				
0	100 (10/10)	0 (0/10)	0 (0/10)	0 (0/10)
250	90 (9/10)	10 (1/10)	0 (0/10)	0 (0/10)
500	100 (10/10)	40 (4/10)	0 (0/10)	0 (0/10)
24, 48 and 72 h				
0	100 (10/10)	0 (0/10)	0 (0/10)	0 (0/10)
250	100 (10/10)	30 (3/10)	0 (0/10)	0 (0/10)
500	100 (10/10)	30 (3/10)	0 (0/10)	0 (0/10)

[a] $MICLD_{50}$ of the virus.

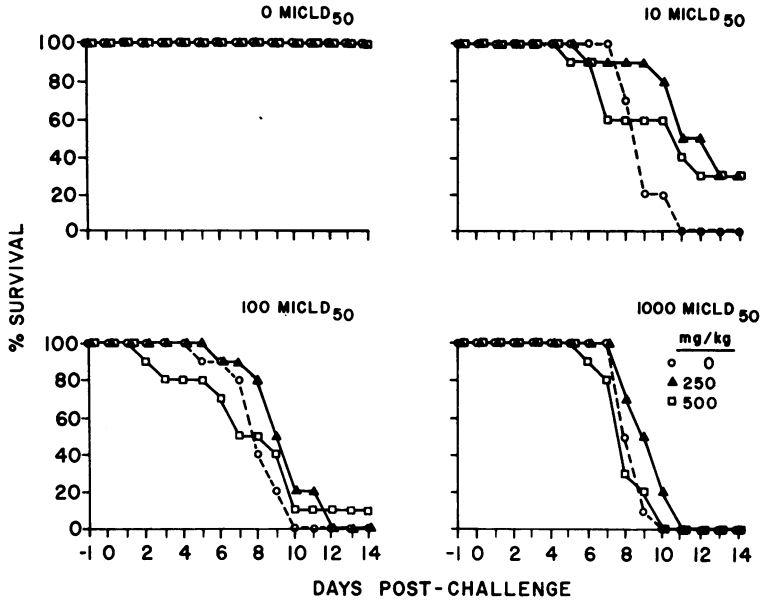

FIG. 3. *Effect of oral treatment of mice with analogue 11,567 at 24, 48, and 72 h post-VEE infection.*

lactically to VEE virus-infected mice. R. W. Krueger and S. Yoshimura (Fed. Proc. 29:635, 1970) reported that a single prophylactic dose of tilorone hydrochloride given 24 h before a lethal s.c. inoculation of Semliki forest virus provided maximal protection. Further, they reported the oral and i.p. mean lethal dose of tilorone to be 959 and 145 mg/kg, respectively. The most uniform increases in the percentage of survivors in the present studies occurred in mice given the compounds orally. When treatment was initiated 24 h after virus inoculation, analogue 11,567 had no significant effect on survival. Consistent with these findings, others have shown that treatment of mice with the optimal oral dose of 250 mg of tilorone hydrochloride per kg 24 to 48 h after a fatal challenge of Semliki forest virus did not significantly change the incidence of mortality (9).

Although the antiviral activity of tilorone hydrochloride has been attributed to induction of interferon (1, 10), exceptions have been reported with several viruses. Giron et al. (3) reported that protection of mice with tilorone hydrochloride against MM virus is apparently not dependent on interferon induction. In their studies, circulating interferon was not detected with i.p. tilorone doses of less than 150 mg/kg. Others have reported that detectable interferon induced by pyran, polyriboinosinic acid · polyribocytidylic acid and bacterial endotoxin does not correlate with the degree of protection obtained by use of these agents against MM virus (13) and mengovirus (11) infections in mice. Giron et al. (3) concluded that determination of circulating interferon concentration is of limited value in assessing antiviral activity, since protection can be demonstrated without the simultaneous detection of interferon. In contrast, others report that protection of mice against intranasal infection with VSV is directly related to the concentrations of interferon induced by different doses of tilorone (1). It appears that MM virus is much more susceptible to the action of tilorone, since lower doses of this compound that do not induce detectable amounts of circulating interferon are effective against MM virus but not against VSV (3).

Virulent Trinidad strain and attenuated TC-83 strain of VEE virus are sensitive to the antiviral activity of interferon (6). Tilorone and structurally related compounds increase serum interferon levels in rodents but not in monkeys or humans (7; H. E. Kaufman and Y. M. Centifanto, J. Clin. Invest. 50:53, 1971). However, antiviral activity has been shown with tilorone hydrochloride against rubella virus in man (G. M. Schiff, C. C. Linnemann, T. Rotte, G. D. Mayer, and S. Trimble, Clin. Res. 21:882, 1973) and with tilorone and its three analogues against the attenuated TC-83 strain of VEE virus in monkeys (G. D. Mayer, A. C. Hagan, and F. Bray, Fed. Proc. 32:704, 1973). The mechanism of this antiviral activity is unclear. Recent in vitro data (W. L. Pannier, unpublished observations) obtained from tilorone-treated, VEE virus-infected Vero cell cultures have shown that 50 μg of tilorone per ml results

in 50% virus plaque reduction with no evidence of cytopathic effects in these cell cultures. These data suggest that other mechanisms may be involved, since it is reported that interferon cannot be induced in Vero cells (2, 15). Other possibilities for protection in the absence of detectable interferon are the stimulation of cellular immunity, the production of lymphokines, and insensitivity of the interferon assay techniques employed.

The interaction of challenge doses of virus with administration of potential antiviral compounds and the importance of route of administration of these compounds is emphasized in our studies. In the case of tilorone and analogue 11,877, their effectiveness by the i.p. route is marginal at best but is greatly increased when given orally. The importance of route is also evident when a dosage of 250 mg of analogues 11,002 and 11,567 per kg is compared by the oral and i.p. routes: significant protection against 100 $MICLD_{50}$ of VEE virus is conferred by 250 mg of 11,002 per kg only when given orally, whereas 250 mg of 11,567 per kg is significantly protective against 1,000 $MICLD_{50}$ of VEE virus only when given i.p.

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