culture which could be replaced by the proteolytic enzyme trypsin points to the important role of proteases in growth.

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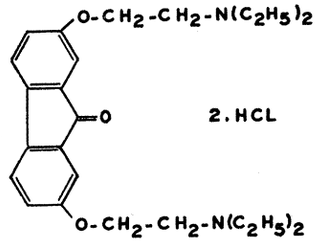
Tilorone Hydrochloride: An Orally Active Antiviral Agent

Abstract. *Tilorone hydrochloride, 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride, given orally to mice before they are infected is active against at least nine viruses of both RNA and DNA groups. The compound is effective when given prophylactically; the optimum time of treatment depends on the route of infection.*

Tilorone hydrochloride, the orange, water-soluble dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one, is a broad-spectrum, orally active antiviral agent.



This compound is active against diverse pathologic conditions. The compound was administered by gavage in aqueous 0.15 percent hydroxyethyl cellulose to CF-1 male mice (15 to 18 g). The animals, in groups of 10 to 30, were observed for 10 days after they were inoculated with virus. Best results were observed against Semliki Forest virus, which is an RNA virus of the arbovirus group; treated mice were completely protected from lethal infections by virus preparations inoculated subcutaneously into the groin (0.1 ml). Although few treated mice survived the severe challenge of vesicular stomatitis virus inoculated intracranially (0.03 ml in the temporal region), the survival time was increased. Deaths from subcutaneously inoculated picornaviruses, and encephalomyocarditis and Mengo viruses can be largely averted by one prophylactic oral dose of the compound.

Infections caused by intraperitoneal challenges (0.1 ml) of the DNA virus herpes simplex were significantly tempered in mice treated orally with tilorone, especially if treatment was begun at least 24 hours before inoculation and continued daily for 7 days. Respiratory infections from intranasal instillation (0.05 ml) of the RNA myxoviruses influenza B (Massachusetts), influenza A/equine-1 (Prague), and influenza A_2 (Jap/305) appear to be least responsive to treatment with tilorone although the survival times of treated mice were extended.

Lung consolidation provoked by influenza A_2 (Jap/305) was significantly diminished in mice treated orally with 250 mg/kg 24 hours before intranasal challenge. However, lung lesions induced either by influenza A_0 (PR_8) virus or lipopolysaccharide did not respond to oral treatment. Furthermore, the course of infection in treated mice inoculated intranasally with lethal doses of influenza A_0 (PR_8) remained unaltered.

Tail lesions induced by subcutaneous inoculation of vaccinia (*1*), a DNA virus, were conspicuously reduced in mice treated daily for 7 days beginning 1 day before challenge. The tails were inoculated subcutaneously (0.1 ml) with 5 ID_{50} of virus (infective dose for 50 percent of the mice). The severity of the lesions was rated subjectively 8 days after infection. A numerical scoring system, that ranged from no lesion (0) to a maximum condition (3), was used. Lesion scores from groups of 20 mice were averaged. Tilorone was more active than *N*-methylisatin-β-thiosemicarbazone under these test conditions. The average tail-lesion score for nontreated mice was 2.00. Mice treated with 250, 100, and 50 mg/kg had average tail-lesion scores of 0.20, 0.74, and 1.40, respectively. Mice treated orally with 250 mg of *N*-methylisatin-β-thiosemicarbazone per kilogram of body weight had an average tail-lesion score of 0.90.

Against encephalomyocarditis virus, activity was demonstrated when tilorone was given orally 48 hours before challenge (Table 2). However, optimum activity was observed when the compound was given 24 hours before virus inoculation. Doses given 3 hours before or after inoculation were weakly active,

Table 1. Oral activity of tilorone hydrochloride (250 mg/kg per dose) against lethal virus infections in mice. The compound was administered before infection at the times indicated. Deaths were recorded over a 10-day period. Survivors were given a score of 11 in calculations of the mean day of death. Groups of 10 to 30 mice were used. Abbreviations: SC, subcutaneous; IC, intracranial; IN, intranasal; IP, intraperitoneal; SF, Semliki Forest; VSV, vesicular stomatitis; EMC, encephalomyocarditis; HS, herpes simplex; T, treated; C, control.

Virus	Route	LD_{50}	Tilorone (hr)	Survivors (%)		Day of death (mean)		*P**
				T	C	T	C	
SF	SC	32	24	100	0	11.0	6.5	<.001
VSV	IC	252	48, 24	20	0	7.2	4.0	<.001
EMC	SC	18	22	80	0	10.1	4.7	<.001
Mengo	SC	39	24	80	5	10.2	5.6	<.001
Influenza	IN							
B (Mass.)		21	24	50	10	9.8	8.0	.01
A/Equine-1		21	24	40	10	9.5	7.8	.01
A_2 (Jap/305)		4	24	30	10	8.9	7.1	.01
HS	IP	63	24, 2†	45	0	10.0	8.5	<.001

* Probability value (Student's *t*-test; 2-tailed). † Plus once a day for 6 days after inoculation.

Table 2. Effect of a single oral dose (250 mg/kg) of tilorone hydrochloride on encephalomyocarditis infections in mice. Mice were inoculated subcutaneously with 8 LD_{50} of virus (lethal dose for 50 percent of the mice). Survivors were determined 10 days after infection.

Treatment time (hr)	Day of death (mean)	Survivors (%)
	Before infection	
48	8.2	40
24	10.6	90
3	5.7	0
	After infection	
3	5.6	0
24	4.0	0
48	4.5	0
	Infected controls	
	4.4	0

as indicated by an increase in the mean day of death. Tilorone given 24 or 48 hours after infection was inactive.

Groups of 20 mice were treated orally with tilorone to determine the optimum time of treatment against Semliki Forest virus. Mice treated with 125 mg/kg 72, 48, 24, or 2 hours before subcutaneous virus challenge (34 LD_{50}, lethal dose of virus which kills 50 percent of animals) had 15 percent, 40 percent, 100 percent, and 20 percent survivors, respectively. Mice treated with 250 mg/kg at 96, 72, 48, 24, or 6 hours before intracranial virus challenge (24 LD_{50}) had 20 percent, 40 percent, 80 percent, 40 percent, and 0 percent survivors, respectively. Thus, the optimum time of treatment depends on the route of virus challenge.

A single oral dose of 100 mg/kg of tilorone, given 24 hours before infection, totally protected male CFE rats (170 g) from paralysis by subcutaneous inoculations of Semliki Forest virus, whereas 70 percent of infected controls were affected.

The LD_{50} for a single dose orally administered to mice is 959 mg/kg, and intraperitoneally administered it is 145 mg/kg. In rats the LD_{50} for a single oral dose is 852 mg/kg, and for a single intraperitoneal dose it is 244 mg/kg.

Tilorone hydrochloride has the broadest spectrum of any orally active antiviral agent yet reported.

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3. We thank S. Yoshimura for technical assistance.

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Tilorone Hydrochloride: Mode of Action

Abstract. *An antiviral serum component is found in mice treated orally with tilorone hydrochloride. The active material fulfills sufficient biological criteria to be classified as an interferon.*

Tilorone hydrochloride is a broad-spectrum antiviral agent active in vivo (*1*). Oral administration stimulates mice to make a protein with the biologic characteristics of interferon.

The compound, dissolved in aqueous 0.15 percent hydroxyethyl cellulose, was administered by gavage to CF-1 male mice weighing 15 to 18 g. To determine the antiviral activity of the serum samples collected by orbital bleeding, pooled serum from groups of at least ten mice was diluted in Eagle's basal medium containing Earle's salts (EBME) to which fetal calf serum (2 percent) and sodium bicarbonate (5 percent) were added. One milliliter of diluted test serum was added to each of six test tubes that contained confluent monolayers of mouse L929 cells. After incubation at 37°C for 24 hours, the serum was removed and the cells were washed twice with Hanks balanced salt solution. One milliliter of vesicular stomatitis virus (VSV) preparation, diluted in EBME to the challenge concentrations, was added to four of the six tubes; the remaining two tubes received only 1 ml of EBME and served as controls. After a 2-hour adsorption period, the cells were washed once with the Hanks solution, and 1 ml of EBME was added. The protective serum dilution (PSD_{50}) represents the dilution of serum that protected 50 percent of the cells from viral cytopathology after 48 hours. Unless stated otherwise, this was the method of assay. Active serum used for interferon characterization was collected from mice 20 to 30 hours after oral treatment with tilorone (250 mg/kg).

Table 1. Effect of enzyme exposure, dialysis, and low *p*H on active mouse serum. Serum titrations were conducted against vesicular stomatitis virus in mouse L929 cells with 50 percent cytopathology as the endpoint.

Treatment	Time of exposure	Serum titer
Trypsin	24 hr, 37°C	$<1:50$
No trypsin		1 : 3200
Ribonuclease	20 hr, 37°C	1 : 800
No ribonuclease		1 : 800
Dialyzed	24 hr	$>1:2500$
Not dialyzed		$>1:2500$
Acidified to *p*H 2.0	24 hr, 4°C	1 : 2560
Not acidified		1 : 2560

The logarithmic curve of the reciprocal of the serum dilution shows that antiviral activity appears as early as 12 hours, reaches a peak at 24 hours, and essentially disappears within 48 hours after oral treatment (Fig. 1).

Serum collected from mice 24 hours after oral treatment with different concentrations of tilorone was titrated on mouse L929 cells against 32 TCD_{50} (tissue culture dose of virus that destroys 50 percent of cells) of vesicular stomatitis virus. A dose response relative to detectable antiviral activity of mouse serum was observed (Fig. 2). The PSD_{50} ranged from a dilution of 1:15 with a dose of tilorone of 5 mg/kg to more than 1:10,000 with a dose of 250 mg/kg. A plateau of stimulation was reached between 250 and 500 mg/kg.

A 1:20 dilution of active serum, that completely protected mouse L929 cells from the cytopathology caused by encephalomyocarditis virus and vesicular stomatitis virus, failed to protect human epithelial (HEp-2) or primary rat embryo cells from the cytopathic effects of these viruses. The cytopathic effects caused by 32,000 TCD_{50} of yellow fever virus in mouse L929 cells were also prevented by this serum.

The effects of enzymes, dialysis, and low *p*H on active mouse serums are seen in Table 1. A 10 percent solution of active mouse serum diluted in EBME was incubated with 0.01 percent trypsin for 24 hours at 37°C. After soybean trypsin inhibitor was added (0.02 percent final concentration), the antiviral activity of the trypsin-digested serum was compared with that of undigested serum containing the soybean inhibitor. The PSD_{50} of the digested serum was less than 1:50 (lower dilutions not tested), whereas that of the undigested serum was 1:2400. Trypsin digestion at 37°C for 1 hour destroyed 95 percent of the antiviral potential of active mouse serum. Exposure of active mouse se-