Antiviral Activity of 10-Carboxymethyl-9-Acridanone

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Intraperitoneal administration of 10-carboxymethyl-9-acridanone sodium salt (CMA) protected at least 50% of mice tested from otherwise lethal infections with Semliki forest, coxsackie B1, Columbia SK, Western equine encephalitis, herpes simplex, and pseudorabies viruses. The protective effect against influenza A2/Asian/J305 and coxsackie A21 viruses was less but was statistically significant. When administered either subcutaneously or orally, CMA protected at least 50% of mice against Semliki forest and pseudorabies viruses; the effect against coxsackie B1 and herpes simplex viruses was less but was statistically significant. Initiation of treatment could be delayed from 2 to 24 h after infection of mice with coxsackie B1, herpes simplex, Semliki forest, and Western equine encephalitis viruses without loss of an antiviral effect. CMA did not inactivate Semliki forest or coxsackie B1 viruses on contact and was without effect against any of the viruses tested in tissue culture by the tube dilution assay. The humoral antibody response in mice to both influenza virus and sheep erythrocytes was unaffected by CMA. After administration of CMA, an interferon-like substance was induced in mice or mouse cell culture but not in rabbits or rabbit cell culture.

The currently available antiviral substances (e.g., methisazone, idoxuridine, and amantadine hydrochloride) all have disadvantages such as limited spectrum of activity, limited therapeutic usefulness, or toxicity. Any new agent which would overcome any or all of these drawbacks would be of interest.

During the course of testing compounds for antiviral activity, one agent, 10-carboxymethyl-9-acridanone, was found to exert appreciable activity against Columbia SK, herpes, and coxsackie B1 virus infections in mice (R. I. Fryer and E. Grunberg, U.S. Patent 3,681,360, 1972). The activity in these experimental infections, which was primarily prophylactic in nature, was sufficient and the spectrum broad enough to warrant further testing of this compound. Therefore experiments were undertaken to determine: (i) the effect against additional viruses, (ii) whether it was therapeutically as well as prophylactically active at doses below the toxic level, (iii) the mechanism of action, and (iv) the effect on humoral antibody synthesis. The results of these studies are described in the present communication.

MATERIALS AND METHODS

Compound description. 10-Carboxymethyl-9-acridanone sodium salt (CMA) is an odorless, yellow, crystalline solid whose melting point is >350 C. The substance is soluble in water (>1%) but not in

acid and its molecular weight is 275.25. CMA has the structural formula shown in Fig. 1.

Acute toxicity. The acute toxicity of CMA was determined in 18- to 20-g Royalhart mice by administering a single dose of the substance either intraperitoneally (i.p.), subcutaneously (s.c.), or orally (p.o.). All animals were observed for a period of 72 h, and the mean lethal dose (LD_{50}) was calculated by the method of Reed and Muench (6).

Virus infections in mice. Swiss albino mice (Royalhart) weighing 9 to 12 g (weanling) were infected i.p. with coxsackie A21, herpes simplex, pseudorabies, and Western equine encephalitis viruses, whereas intranasal instillation under light ether anesthesia was used to infect weanling mice with influenza A2/Asian/J305 and Sendai (parainfluenza type 1) viruses. Mice weighing 18 to 20 g were infected i.p. with Columbia SK, coxsackie B1, and Semliki forest viruses. All animals received approximately 10 LD_{50} of the appropriate virus.

Treatment. CMA was dissolved in water and administered i.p., s.c., or p.o. 24 h before virus infection, immediately after virus infection, and 24 h after virus infection for all virus infections in mice except influenza and Sendai. Mice infected with in-
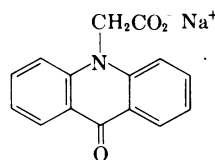

$$CH_2CO_2^- \ Na^+$$

FIG. 1. *Structural formula of CMA.*

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fluenza and Sendai viruses received seven i.p. treatments (at 0, 1, 5, 24, 30, 48, and 72 h relative to virus infection at 0 h). Adult mice received 1.0 ml of the desired doses of CMA or water, whereas weanling mice were given 0.5 ml. The mean protective dose (PD_{50}) was calculated by the method of Reed and Muench (6), whereas statistical significance, when PD_{50} values could not be obtained, was determined by the Fisher exact test.

In vitro tube dilution assay. Monolayers of WI-38 and rhesus monkey kidney cells were infected with serial 10-fold dilutions of coxsackie B1, herpes simplex, influenza A2/Asian, rhinovirus 42, Semliki forest, and vaccinia viruses. The mean tissue culture infective dose of the virus-infected cultures in the presence and absence of 1,000 μg of CMA per ml (the maximum tolerated noncytotoxic dose for tissue culture cells) was determined on the basis of cytopathogenic effect after 7 days of incubation for all viruses except influenza which was assayed by the hemadsorption technique 4 days after virus infection.

Contact inactivation. Viruses (coxsackie B1, Semliki forest) were incubated for 2 h at room temperature in the presence of 1,000 μg of CMA per ml or diluent alone (phosphate-buffered saline, pH 7.2, containing 0.5% bovine albumin). Serial 10-fold dilutions of virus control and virus plus drug mixtures were injected i.p. into mice and the LD_{50} values were determined (6).

Humoral immune response. Swiss albino mice weighing 18 to 20 g received 0.2 ml i.p. of CMA at 100 mg/kg either once daily for 3 days before immunization or once daily for 7 days after immunization. Antigens used were influenza A2/Asian/J305 virus or sheep erythrocytes. Mice received 0.1 ml i.p. of influenza virus (hemagglutination titer = 2,048 units/ml) or 0.2 ml i.p. of a 1% suspension of sheep erythrocytes. Plasma from drug-treated and water-treated mice was obtained either at days 7, 14, and 21 (influenza virus) or at day 14 (sheep erythrocytes) postimmunization and the hemagglutination inhibition (HI) or hemagglutination (HA) titers of the respective plasma samples were determined against the appropriate antigen.

Induction of interferon-like activity. Swiss albino mice weighing 18 to 20 g received 1.0 ml i.p. of CMA at 200 mg/kg. Animals were sacrificed at 2, 6, and 24 h after treatment and pooled sera were tested for interferon-like activity in monolayers of mouse L cells and rabbit (RK-13) cells. Monolayers were incubated for 18 h in the presence of various serum dilutions before challenge with approximately 100 plaque-forming units of vesicular stomatitis virus. Interferon-like activity titer was defined as the reciprocal of the highest serum dilution that caused a 50% reduction in plaque-forming units.

Rabbits received 1.0 ml intravenously of CMA at 4,000 μg/ml and were bled at 2, 6, and 24 h. The serum samples were tested for interferon-like activity in monolayers of RK-13 cells as described above for mouse sera.

In vitro induction of an interferon-like activity was determined in monolayers of mouse L cells and rabbit RK-13 cells which were incubated in the presence of various concentrations of CMA for 18 h and subsequently challenged with vesicular stomatitis virus.

RESULTS

Acute toxicity. The LD_{50} values obtained after a single administration of CMA were: 1,516 mg/kg i.p., 3,031 mg/kg s.c., and >4,000 mg/kg p.o.

In vivo prophylactic activity. Appreciable activity was observed against Semliki forest, coxsackie B1, herpes simplex, and pseudorabies virus infections in mice when CMA was administered i.p. at −24, 0, and 24 h relative to virus infection at 0 h (Table 1). CMA was somewhat less active against Columbia SK virus. In the case of Western equine encephalitis virus, the PD_{50} was <100 mg/kg and the end point was not determined.

Utilizing the −24-, 0-, 24-h schedule and administering CMA s.c., a definite antiviral effect against Semliki forest and pseudorabies viruses was observed. Oral administration of CMA was also effective using the same treatment schedule, but considerably higher levels of CMA were necessary to obtain a 50% protection end point against Semliki forest and pseudorabies viruses.

In Table 2 are listed those viruses for which PD_{50} values could not be obtained but against which CMA exerted a statistically significant effect ($P \leq 0.05$) when the test substance was

TABLE 1. *Activity of CMA against virus infections in mice*

Virus[a]	PD_{50} (mg/kg)[b]
Ribonucleic acid	
Semliki forest	18 i.p.
	47 s.c.
	320 p.o.
Coxsackie B1	36 i.p.
Columbia SK	105 i.p.
Western equine encephalitis ..	<100 i.p.
Deoxyribonucleic acid	
Herpes simplex	60 i.p.
Pseudorabies	17 i.p.
	154 s.c.
	530 p.o.

[a] Weanling (9 to 12 g) mice were infected i.p. with herpes simplex, pseudorabies, or Western equine encephalitis viruses. Adult (18 to 20 g) mice were infected i.p. with coxsackie B1, Semliki forest, or Columbia SK viruses. Approximately 10 LD_{50} of virus was used for all infections.

[b] Treatment: Weanling mice received 0.5 ml and adult mice received 1.0 ml of CMA i.p., s.c., or p.o. at 24 h before virus infection, immediately after virus infection, and 24 h after virus infection.

administered i.p., s.c., or p.o. CMA produced an effect against coxsackie A21 and influenza viruses when administered i.p., whereas s.c. or p.o. treatment was effective against herpes simplex and coxsackie B1 virus infections in mice. The i.p. administration of CMA at 50 mg/kg was without effect against Sendai virus infection.

In vivo therapeutic activity. The therapeutic effects of CMA after i.p. treatment of mice infected with coxsackie B1, herpes simplex, Semliki forest, and Western equine encephalitis viruses are shown in Table 3. The time of initiation of therapeutic treatment varied from immediately after virus infection to 48 h after virus infection. Mice receiving treatment beginning 24 h before virus infection served as prophylactic controls. All mice received three i.p.

TABLE 2. *Effect of i.p., s.c., or p.o. treatment with CMA against virus infections in mice*

Virus[a]	Dose (mg/kg)[b]	No. of mice surviving/no. tested at 21 days		Corrected survival (%)[c]	P[d]
		Treated	Control		
Coxsackie A21	100 i.p.	10/24	2/24	29	0.024
Influenza A2/Asian	50 i.p.	5/20	0/23	25	0.016
Herpes simplex	200 s.c.	16/48	3/47	27	<0.001
	800 p.o.	9/31	1/32	26	0.005
	400 p.o.	10/47	3/47	15	0.027
Coxsackie B1	200 s.c.	12/39	1/37	28	0.001
	400 p.o.	7/32	1/31	19	0.029

[a] Weanling mice were infected i.p. (coxsackie A21, herpes simplex) or intranasally (influenza); adult mice were infected i.p. with coxsackie B1 virus.

[b] Treatment was as described in Table 1 with the exception that influenza virus-infected mice received 0.5 ml i.p. of CMA at 0, 1, 5, 24, 30, 48, and 72 h relative to virus infection at 0 h.

[c] Percentage of survival in treated mice less percentage of survival in control.

[d] Fisher exact test.

TABLE 3. *Effect of different i.p. treatment schedules with CMA against virus infections in mice*

Virus[a]	Time (h) of drug administration[b]	No. of mice surviving/no. tested at 21 days		Corrected survival (%)	P
		Treated	Control		
Coxsackie B1	−24, 0, +24	37/48	4/48	69	<0.001
	0, +24, +48	38/48	4/48	71	<0.001
	+6, +24, +48	34/40	3/40	77	<0.001
	+12, +24, +48	20/32	2/32	56	<0.001
	+18, +24, +48	2/16	2/16	0	>0.05
Herpes simplex ..	−24, 0, +24	27/40	1/40	65	<0.001
	0, +24, +48	20/40	1/40	47	<0.001
	+6, +24, +48	17/40	1/40	40	<0.001
	+24, +48, +72	10/48	1/48	19	0.004
	+48, +72, +96	0/8	0/8	0	>0.05
Semliki forest	−24, 0, +24	36/39	4/40	82	<0.001
	0, +24, +48	26/39	4/40	57	<0.001
	+2, +24, +48	21/48	7/47	29	0.002
	+6, +24, +48	9/39	7/40	6	>0.05
Western equine encephalitis	−24, 0, +24	25/32	7/32	56	<0.001
	0, +24, +48	15/31	7/32	27	0.026
	+6, +24, +48	17/32	7/32	31	0.009

[a] Virus infection, see Table 1.

[b] Treatment: 1.0 ml i.p. for coxsackie B1 and Semliki forest; 0.5 ml i.p. for herpes simplex and Western equine encephalitis. Times of drug administration are relative to virus infection at 0 h. CMA was tested at 50 mg/kg (coxsackie B1, Semliki forest) and 100 mg/kg (herpes simplex, Western equine encephalitis).

treatments. From the results shown in Table 3, it can be seen that in the case of coxsackie B1-infected mice, essentially the same protective effect was obtained when CMA was administered prophylactically at 24 h before virus infection, immediately after virus infection, and again at 24 h after virus infection (−24, 0, 24 h), or immediately after virus infection, 24 h after virus infection, and again at 48 h after virus infection (0, 24, 48 h). However, administration of CMA at 0, 24, and 48 h to mice infected with herpes simplex, Semliki forest, or Western equine encephalitis virus was somewhat less effective than administration by the −24-, 0-, 24-h treatment schedule.

It was possible to delay the initiation of treatment for 12 h in mice infected with coxsackie B1 virus and still maintain an appreciable level of activity (56% survival).

When the start of treatment with CMA was delayed for 6 h after infection with herpes simplex virus, significant protection (40% survival), which was similar to that observed when treatment was initiated immediately after infection (47% survival), was obtained. Even when therapy was not initiated until 24 h after virus infection, the level of protection (19% survival) was statistically significant.

In the case of Semliki forest virus, appreciable activity was still seen when treatment with CMA was initiated at 0 h (57% survival), although there was a reduction of the protective effect over that seen when treatment was started 24 h before virus infection (82% survival). Delaying the start of treatment with CMA to 2 h after virus infection further reduced the protective effect (29% survival), but the results were still statistically significant.

In the case of Western equine encephalitis virus, although some loss in protective effect was observed if treatment was started immediately after virus infection (27% survival) instead of 24 h before virus infection (56% survival), a further loss in antiviral effect was not observed when treatment with CMA was begun 6 h after virus infection (31% survival). The effect of delaying treatment for longer periods of time after infection with Western equine encephalitis virus was not determined.

Effect of CMA on growth of viruses in tissue culture. The in vitro antiviral activity of CMA against coxsackie B1, herpes simplex, influenza rhinovirus, Semliki forest, and vaccinia viruses is presented in Table 4. Activity in the tube dilution assay used was defined as a difference of at least two logarithms between the mean tissue culture infective dose of virus control and drug-treated cells. As shown, CMA was with-

out antiviral effect in vitro against any of the viruses tested.

Contact inactivation. Incubation of Semliki forest or coxsackie B1 virus for 2 h at room temperature in the presence of 1,000 μg of CMA per ml did not significantly reduce the infectivity of these viruses in mice (Table 5).

Humoral immune response. The effects of treatment with CMA on the humoral antibody response in mice administered either influenza virus or sheep erythrocytes are shown in Table 6. Results obtained with a known immunosuppressive agent (methotrexate) are also listed for comparison. When CMA or methotrexate was administered to mice before immunization (3 the HI or HA titers of plasma from drug-treated mice were essentially similar to those of water-treated mice. When CMA was administered after immunization (7 Post), the plasma HI or HA titers were not significantly different from those of the water-treated controls, whereas the

TABLE 4. *In vitro antiviral effect of CMA*[a]

Virus[b]	$\Delta Log_{10} TCID_{50}$ of virus control–drug treated[c]
Coxsackie B1	0.0
Herpes simples	0.0
Influenza A2/Asian	0.7
Rhinovirus 42	0.2
Semliki forest	0.3
Vaccinia	0.0

[a] Tested at 1,000 μg/ml.

[b] Coxsackie B1 and influenza A2/Asian viruses were grown in rhesus monkey kidney cells, whereas herpes simplex, rhinovirus 42, Semliki forest, and vaccinia viruses were grown in WI-38 cells.

[c] Activity was defined as a difference of at least 2 logarithms between the mean tissue culture infective dose (TCID$_{50}$) of the virus control and drug-treated cells.

TABLE 5. *Effect of in vitro contact with CMA on the infectivity of Semliki forest and coxsackie B1 viruses in mice*

Virus[a]	$Log_{10} LD_{50}$		Δ Log	Virus inactiva-tion[b]
	Virus control	Virus + CMA		
Semliki forest .	6.2	5.3	0.9	—
Coxsackie B1 ..	3.9	4.5	0.0	—

[a] Virus control and virus plus drug (final concentration 1,000 μg/ml) were incubated at room temperature for 2 h before assay for infectivity in mice.

[b] Virus inactivation was defined as a difference of at least 2 logarithms between the LD$_{50}$ of the virus control and virus plus drug.

TABLE 6. *Effect of CMA on the humoral immune response in mice*

Treatment schedule	Antigen[a]	Drug[b]	Reciprocal of plasma titer at day:		
			7	14	21
3 Pre[c]	Influenza virus	Water	320[d]	320	160
		CMA	160	160	160
		Methotrexate	320	320	320
	Sheep erythrocytes	Water		160[e]	
		CMA		80	
7 Post[f]	Influenza virus	Water	320[d]	320	640
		CMA	320	320	640
		Methotrexate	40	40	Not done
	Sheep erythrocytes	Water		320[e]	
		CMA		640	
		Methotrexate		20	

[a] Mice received 0.1 ml i.p. of influenza A2/Asian/J305 virus (hemagglutination titer = 2,048 units/ml) or 0.2 ml i.p. of a 1% suspension of sheep erythrocytes.

[b] CMA tested at 100 mg/kg; methotrexate tested at 2 mg/kg.

[c] Treated mice received 0.2 ml of the test substance or water i.p. once daily for 3 days before administration of antigen.

[d] HI titer.

[e] HA titer.

[f] Treated mice received 0.2 ml of the test substance or water i.p. 2 h after administration of antigen and once daily thereafter for a total of seven treatments.

expected suppression of the HI or HA titers of methotrexate-treated mice was observed.

Interferon-like activity. The results of testing pooled sera from mice given a single i.p. treatment with CMA or poly(rI:rC) for interferon-like activity are shown in Table 7. When mouse sera were assayed in the homologous mouse L cells, interferon titers of both CMA-treated and poly(rI:rC)-treated animals were highest at 2 and 6 h and barely detectable at 24 h. The activity of both pools of sera was low

TABLE 7. *Induction of an interferon-like substance in mice by CMA*

Compound[a]	Time (h) post-injection	Titer[b]	
		Mouse L cells	Rabbit RK-13 cells
CMA	2	1,200	94
	6	790	<20
	24	20	<10
Poly(rI:rC)	2	1,365	<10
	6	810	33
	24	38	<10

[a] Mice weighing 18 to 20 g received a single i.p. treatment with CMA (200 mg/kg) or poly(rI:rC) (6.25 mg/kg). Animals were killed at 2, 6, and 24 h after drug administration, and the serum was tested for interferon-like activity in monolayers of mouse L cells and rabbit RK-13 cells by a plaque reduction assay with vesicular stomatitis virus.

[b] Units per 2 ml of reaction volume.

TABLE 8. *In vitro induction of an interferon-like activity in cell culture[a]*

Cell line	Concn (μg/2 ml) resulting in 50% reduction (PFU)	
	CMA	Poly(rI:rC)
Mouse L	57	1.3
Rabbit RK-13	>2,000	9.3

[a] Monolayers of mouse L cells and rabbit RK-13 cells were incubated for 18 h in the presence of various concentrations of CMA or poly(rI:rC) and subsequently challenged with approximately 100 plaque-forming units (PFU) of vesicular stomatitis virus.

when tested in the heterologous rabbit RK-13 cells.

Intravenous injection of 4,000 μg of CMA into rabbits failed to induce detectable levels of an interferon-like substance in the serum.

The protective effect resulting from incubation of mouse L cells or rabbit RK-13 cells with CMA or poly(rI:rC) before infection with vesicular stomatitis virus can be seen in Table 8. CMA induced a protective effect in mouse L cells but not in rabbit RK-13 cells, whereas poly(rI:rC) afforded protection to both cell types.

DISCUSSION

CMA is structurally related to several acridine derivatives which have been shown to

possess antiviral properties (1–5, 7), and two of these substances, mepacrine and Acranil, have recently been described as inducers of an interferon-like substance in mice (1).

The results of the present study indicate that CMA is prophylactically active (PD_{50}) against both ribonucleic acid and deoxyribonucleic acid virus infections in mice at doses well below the toxic level when treatment is administered by the i.p., s.c., or p.o. route. Of particular importance is the observation that the substance also exerts a definite therapeutic effect in certain virus infections. CMA was shown to provide a definite protective effect when treatment was delayed for 2 h (Semliki forest), 6 h (Western equine encephalitis), 12 h (coxsackie B1), or even 24 h (herpes simplex) after viral infection.

The activity found in mouse sera after i.p. administration of CMA has been described as interferon like. It appears to be species specific but, unlike poly(rI:rC), CMA does not induce an interferon-like substance in rabbits. Other characteristics normally associated with interferon need to be examined to more fully characterize this substance. It is possible that the antiviral effect noted with CMA is a composite of interferon-like activity and other, at present, undefined factors. The observation that CMA is therapeutically effective casts some doubt that interferon-like activity is the only explanation for its antiviral effect.

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