PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1114–1119 (1974)

Oral Induction of Interferon by a Low-Molecular-Weight Compound (37963)

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(Introduced by Morris Pollard)
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Prior to 1970, the only known interferon inducers with a low molecular weight were cycloheximide and kanamycin (1). That year Krueger and Mayer (2) (3) reported interferon induction after oral administration of another low-molecular-weight compound, tilorone hydrochloride. Tilorone-induced interferon was effective against a variety of RNA and DNA viruses. Subsequently, DeClercq and Merigan (4) found that while oral treatment was the route of choice, tilorone injected intraperitoneally was also effective. The findings of these investigators that a polycyclic aromatic compound was an effective inducer of interferon prompted us to test compounds of similar structure. Among those tested, one compound induced significant levels of antiviral activity when administered orally to mice. This report presents the results of our investigation of the interferon-inducing and antiviral potential of bis-ω-piperidinylacetyldibenzofuran·HCl (MA-56).

Materials and Methods. MA-56 was prepared and supplied by our Medicinal Chemistry Department. It has a molecular weight of 491, and was originally synthesized in 1936 by Tomita (5). This dibenzofuran is a white crystalline substance readily soluble in water and stable in acidic solution. Tilorone hydrochloride was supplied through the courtesy of Dr. R. F. Krueger of the W. S. Merrell Co., Cincinnati, OH. Cycloheximide and actinomycin D (Act D) were purchased from CalBiochem, Los Angeles. Soybean trypsin inhibitor was obtained from the Research Products Division, Miles Laboratories, Kankakee, IL.

Male CFW mice (18–20 g) were obtained from Carworth Farms, New City, NY and used for interferon induction studies. For the animal protection experiments and cycloheximide studies performed in this laboratory, CFW/Lob mice obtained from the Lobund Laboratory of Notre Dame were used. Mouse protection studies done at the Plum Island Laboratories of the USDA were performed with d/o strain mice.

Solutions of the test compounds in 0.85% saline were prepared just prior to use and administered by gavage. Food and water were supplied *ad lib.* Unless specified otherwise, the mice were bled 16–20 hr after treatment by severing the axillary artery. Serum was harvested within 2 hr, filtered through a 0.22-nm Millipore filter and stored at 4° until assayed—no longer than 72 hr.

Interferon titers were determined by a plaque reduction assay utilizing mouse L-cells and the Indiana strain of vesicular stomatitis virus (VSV). Twofold serial dilutions of sera were prepared in Eagle's minimal essential medium (MEM) without serum supplement. Assay plates (60-mm plastic petri plates) were seeded with 5×10^5 cells in 4 ml of MEM containing 10% fetal bovine serum (FBS). Seventy-two hr

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later, the confluent monolayers were treated with 2 ml of each dilution of the series, three assay plates per dilution. The diluted serum was removed by aspiration and the cells exposed to 75–100 plaque forming units of VSV for 1.5 hr at 37°. Following infection, an agar overlay (1% Noble agar in MEM, FBS 5%) was applied to the plates which were incubated at 37° for 48 hr. Prior to counting, the plates were treated with neutral red (1:10,000) to facilitate enumeration of the plaques. A unit of interferon is defined as the reciprocal of that dilution at which a 50% reduction in plaque count occurs. In our system, an NIH mouse interferon standard (Catalog No. G002-902-026) diluted to contain 600 U/ml of interferon had an assayed titer of 220 U/ml.

In vivo antiviral activity was determined in our laboratory by intraperitoneal challenge of treated mice with encephalomyocarditis virus (EMC virus) or herpes simplex Type II virus (HSV). The EMC virus was obtained from the American Type Culture Collection, Rockville, MD. HSV is a mouse adapted strain obtained from Dr. R. W. Darlington, St. Jude's Hospital, Memphis, Tennessee. Dr. T. Merigan kindly tested MA-56 for its ability to protect against intranasal challenge with VSV using previously reported procedures (4). Drs. J. Y. Richmond and C. H. Campbell, Plum Island Animal Disease Laboratory, Greenport, NY, collaborated with us by carrying out animal protection experiments to test the protective effect of MA-56 against intraperitoneal challenge with foot and mouth disease virus (FMDV).

A variety of standard physical and biological tests were used to characterize the active principle as an interferon.

Results. MA-56 induced antiviral activity. Eighteen hours after the oral administration of MA-56 at a concentration of 500 mg/kg, the serum of CFW mice exhibited significant antiviral activity (1000 U/ml). Intraperitoneal injection of 200 mg/kg resulted in activity approaching 150 U/ml with the mice showing clear signs of toxicity. The activity observed after an intravenous dose of 75 mg/kg was not significant. Direct treatment of mouse L-cell and human foreskin fibroblast monolayers with MA-56 did not induce these cells to produce detectable antiviral activity.

Vesicular stomatitis virus was not inactivated after direct exposure for 1 hr to mouse serum that had assayed at 690 U/ml and such sera did not protect chicken, monkey, and human cells from infection.

Table I shows the results of the tests used to characterize MA-56-induced antiviral activity as an interferon.

Dose response. The antiviral activity of sera from mice treated with MA-56 reaches its maximum after 16–18 hr. Sera were obtained at 18 hr from mice fed various concentrations of this compound and assayed. Optimal antiviral activity was observed within a narrow range, the best response being induced with a dose of 500 mg/kg (Fig. 1).

Inhibition of serum antiviral activity by actinomycin D. Inhibition of cellular RNA synthesis effectively blocks the establishment of an antiviral state in interferon-treated cells (6). Actinomycin D was used in conjunction with serum from MA-56-treated mice and the VSV:mouse L-cell system to determine whether the observed antiviral

TABLE I. Characterization of MA-56-Induced Antiviral Activity as an Interferon.

Characteristic	Test response[a]	
	Control	Treated
Dialysis[b]	660	425
pH stability[c]	890	560
Heat inactivation[d]	755	<10
Trypsin sensitivity[e]	1250	23
Ribonuclease resistance[f]	250	330

[a] Units of antiviral activity/ml serum.

[b] 24-hr dialysis against 0.85% saline.

[c] Serum adjusted to pH 2.0 and held for 24 hr in the cold (4°).

[d] Serum maintained at 56° for 1 hr.

[e] 1 ml of serum added to 8.2 ml of MEM at pH 7.0 was treated with 0.4 ml of 0.25% trypsin at 37° for 24 hr. 0.4 ml of trypsin inhibitor (0.5% solution) was then added.

[f] 0.1 ml of a 0.2% solution of ribonuclease was added to 1.9 ml of serum, then incubated at 37° for 24 hr.

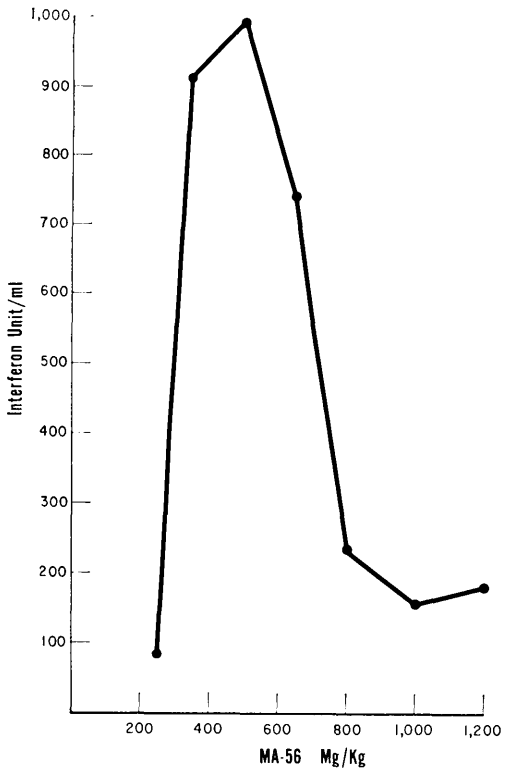

FIG. 1. Antiviral activity of serum 18 hr after the oral administration of various dose concentrations of MA-56.

TABLE II. The Effect of Actinomycin D on Serum Antiviral Activity.[a]

Treatment of cells[b]	Virus titer[c] (PFU/ml)
Virus control	8.2×10^7
Actinomycin D control	7.4×10^6
Serum	5.8×10^4
Actinomycin D added 2 hr *prior to* serum addition	1.6×10^6
Actinomycin D added 3 hr *after* serum addition	1.1×10^5
Actinomycin D added 5 hr *after* serum addition	8.3×10^4

[a] Serum from mice treated with MA-56 was diluted with MEM to contain 350 U of antiviral activity per 2 ml.

[b] 2 ml of the antiviral serum was added to appropriate test plates for 5 hr starting at time 0. Act D (1 μg/ml) treatment was begun at times shown. Plates treated with Act D 2 hr prior to the addition of diluted *normal* mouse serum (time 0) served as the Act D control. All plates were infected with VSV (MOI of ≃1.5) 24 hr after time 0 and virus was harvested 8 hr later.

[c] Titer determined on virus pooled from duplicate plates.

activity of the serum could be inhibited. The results (Table II) follow the pattern expected when antiviral activity is due to interferon.

Effect of cycloheximide. Cycloheximide can be used to differentiate between viral and nonviral induction of interferon (7–9). This compound will inhibit interferon induction by viral agents when animals are pretreated or when it is administered simultaneously with the inducer. With nonviral agents such as endotoxin or synthetic polynucleotides, an enhancing effect is noted. MA-56 seems to act like a viral inducer as interferon induction is completely suppressed in the presence of this inhibitor of protein synthesis (Table III).

Hyporeactive period after interferon induction. Multiple-dose therapy with interferon inducers is hindered because a period of hyporeactivity to the inducing agent occurs after the initial inducing dose. This phenomena is observed following the administration of 500 mg/kg MA-56. In Fig. 2, a typical interferon-induction curve (solid line) is seen following the first dose. Additional treatment (broken line) with the same dose concentration at intervals up to 40 hr after the initial dose failed to induce interferon.

Animal protection. A single 500 mg/kg dose of MA-56 administered within a 24-hr period prior to challenge with virus will provide effective protection against both DNA and RNA viruses (Table IV). An attempt was made to correlate the serum interferon titer with the degree of protection. In a systemic HSV infection, maximum protection was achieved at a serum concentration of 150 U/ml. Serum concentration up to 650 U/ml were without further effect (Fig. 3).

Interferon concentration in tissue. Selected tissues were assayed for antiviral activity after test animals had been administered a single dose of either MA-56 (500 mg/kg) or tilorone (250 mg/kg). All tissue activities were comparable while the

TABLE III. Effect of Cyclohexiimide on Interferon Induction by MA-56.

Treatment	Serum antiviral activity[a] (Hours after treatment)		
	8	16	32
MA-56[b] (400 mg/kg)	63	245	59
Cycloheximide[c] (200 mg/kg)	<20	<40	<16
MA-56[b] (400 mg/kg) plus cycloheximide[c] (200 mg/kg)	<10	<15	<10

[a] Interferon units/ml.
[b] Oral administration.
[c] Intraperitoneal administration.

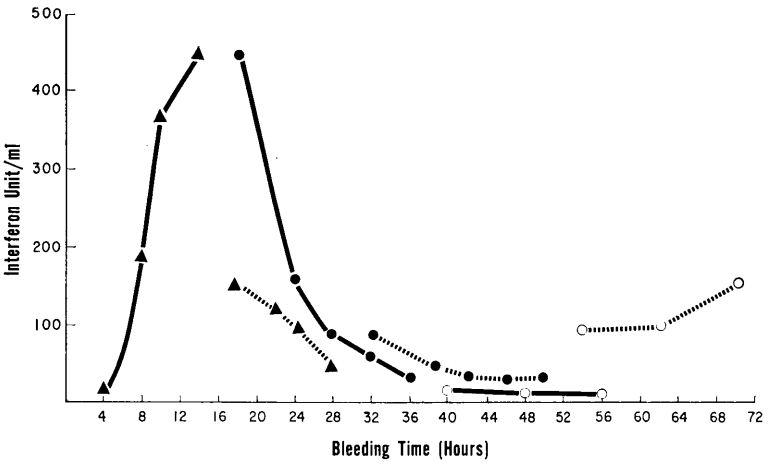

FIG. 2. A composite of three experiments: Expt 1 (▲), Expt 2 (●) and Expt 3 (○) showing the effect of single and double doses of MA-56 on interferon induction. Data from animals receiving a single dose is plotted on solid lines. Data plotted on broken lines shows response of mice receiving a second dose when single-dose mice in the same time group were bled. Mice given a second dose were bled 14 hr later and their response plotted at the hour which represents the total elapsed time from the first dose.

TABLE IV. Effect of a Single Oral Dose[a] of MA-56 upon the Survival of Mice Infected with Selected Viruses.

Challenge virus	Route of infection	Infective dose	Survivors (%)	
			Control	Test
EMC	ip	40 LD_{50}	12	88
EMC[b]	ip	40 LD_{50}	0	100
HSV	ip	50 LD_{50}	0	100
FMDV[c]	ip	80 LD_{50}	14	100
VSV[d]	in	5×10^6 PFU	20	80

[a] 500 mg/kg administered within a 24-hr period prior to viral challenge.
[b] Test animals in this single experiment were 20-g male CFW/Lob germfree mice.
[c] Data from Drs. Richmond and Campbell, Plum Island Animal Disease Laboratory, Greenport, New York.
[d] Data from Dr. Merigan, Stanford University.

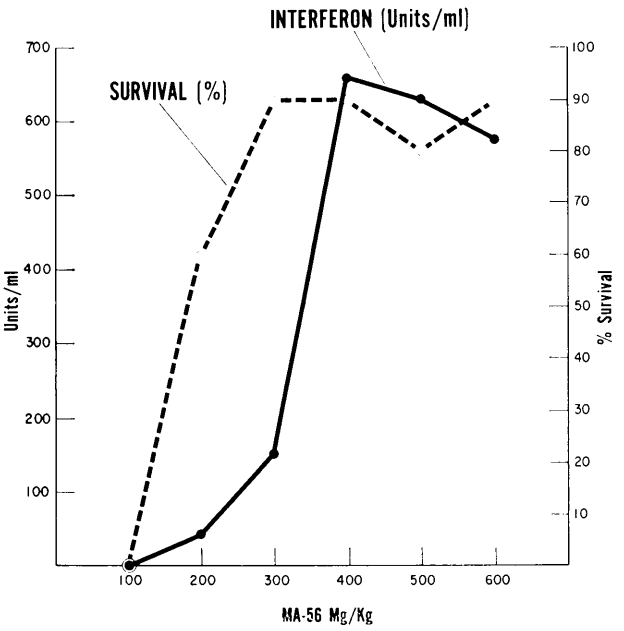

FIG. 3. Comparison of serum interferon titer to the protection induced by MA-56. Groups of 18 mice were used for controls and for each dose level of MA-56. 20 hours after treatment, 10 animals from each group were challenged with 100 LD_{50} of HSV given intraperitoneally. The remaining 8 mice were bled at this time to provide serum for interferon assays.

serum concentration was fourfold higher with tilorone even with a dose concentration one-half that of MA-56 (Table V).

Discussion. The antiviral activity of serum from mice fed MA-56 meets accepted biological and physical criteria for interferon-mediated protection (10). This data provides additional evidence that low-molecular-weight compounds can induce interferon and, as a result, protect against lethal viral challenge. While Sill *et al.* (11) have reported that even minor molecular and structural modifications produce pronounced effects upon interferon stimulation and hyporesponsiveness, there is some similarity between the observations reported here and those published by other investigators on the interferon-inducing activity of tilorone (2, 3). Neither compound is effective as an *in vitro* interferon inducer, but both demonstrate late peaking kinetics which resemble interferon induction by viruses. In these respects, they differ from polyanionic polymeric substances of high molecular weight.

TABLE V. Antiviral Activity in Selected Mouse Tissues After a Single Dose of Low-Molecular-Weight Interferon-Inducing Compounds.

Tissue	Antiviral activity[a]	
	MA-56[b]	Tilorone[c]
Lymph node	63	91
Spleen	39	38
Thymus	23	37
Kidney	31	21
Lung	<25	20
Brain	ND[d]	—[e]
Serum	303[f]	1205

[a] Interferon units/ml of a 20% w/v tissue extract.
[b] 500 mg/kg dose.
[c] 250 mg/kg dose.
[d] None detected.
[e] Not done.
[f] Interferon units/ml serum.

Summary. The antiviral activity induced in mice by MA-56 has been characterized as an interferon. Maximum serum concentration is attained within 16–18 hr after feeding a single oral dose ranging from 400

to 500 mg/kg body wt. MA-56 induces a hyporeactive period of at least 40 hr. Interferon-production kinetics and cycloheximide experiments indicate that MA-56, like tilorone, has an induction pattern that can be blocked by cycloheximide and resembles viral induction rather than that of synthetic polynucleotides and bacterial endotoxin. Although MA-56 apparently does not induce a very high concentration of titratable interferon when compared to other inducers, its ability to protect mice against the lethal challenge of several viruses has been demonstrated.

The authors are indebted to Drs. M. Pollard and T. Merigan for their contributions in the total development of this study. We also thank our collaborators at Plum Island Laboratory for testing FMDV and Anna Huff of our laboratory for her excellent technical assistance.

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Received Oct. 18, 1973 P.S.E.B.M., 1974, Vol. 145.