A NEW ANTIVIRAL AGENT DESIGNATED 6-MFA FROM *ASPERGILLUS FLAVUS*

II. GENERAL PHYSICO-CHEMICAL CHARACTERISTICS OF 6-MFA

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Studies on the physico-chemical characteristics of a new antiviral agent designated 6-MFA obtained from *Aspergillus flavus*, in culture show that 6-MFA is a thermolabile, non-dialyzable, Seitz-filterable and high molecular substance and can be withstand freeze-drying. Biosynthesis of 6-MFA is favored at pH 6. 6-MFA is more stable *in vitro* at pH 6 and 7. Virus-like particles are associated with preparations of 6-MFA.

An antiviral agent designated 6-MFA, produced by a strain of *Aspergillus flavus*, active against Semliki Forest, Chikungunya and neurovaccinia in mice, has been reported from this laboratory[1,2]. The optimal conditions of fermentation, production and bioassay of 6-MFA have also been reported[3]. In this paper pH stability and physico-chemical characteristics of 6-MFA are discussed.

Materials and Methods

Mice. Swiss-CDRI strain mice, 35-days old, weighing 16~18 g were used.

Virus. The Semliki Forest virus (SFV) of SMITHBURN and HADDOW[4] obtained from ATCC, U.S.A., was maintained in mice by intracranial inoculations at intervals of 3 months. Pools of brains from infected animals were collected aseptically and 10 % w/v homogenate in HANKS BSS was prepared. The homogenate was clarified by centrifugation at 3,000 r.p.m. for 30 minutes, and supernatant stored at −10°C in aliquots of 1 ml each. Dilution of the virus was prepared and LD_{50} calculated by REED and MUENCH formula[5], was found to be $10^{-6.62}$, 0.5 ml, subcutaneously per mouse.

6-MFA. It was prepared by acetone treatment of the crude filtrate as described[3].

Antiviral testing *in vivo*. Unless otherwise stated, mice were injected intraperitoneally (i.p.) with 6-MFA prepared in distilled water at the rate of 2 ml per mouse. Mice challenged 24 hours later with SFV, 100 LD_{50}, subcutaneously (s.c.), 0.5 ml per mouse. Control mice received buffer saline instead of 6-MFA and were challenged by SFV similarly. Observations on the appearance of the specific symptoms and mortality were taken daily for 14 days when the experiment was terminated.

Experimental

Effect of Varying pH of the Growth Medium on the Production and Activity of 6-MFA

SHOPE's glucose-nitrate-yeast broth[6] ranging in pH from 2~8 were prepared and flasks seeded with *A. flavus* spore suspension for fermentation and 6-MFA was prepared. Results of antiviral

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assay test (Table 1) indicated that maximum activity (100 % protection) was achieved with preparation of 6-MFA extracted from fungus incubated in medium of pH 6.

pH Stability of 6-MFA *in vitro*

Crude filtrate (1 litre) was prepared as before and was dispensed into 5 flasks, 200 ml each. Chilled acetone (1 : 1) was added and flasks were kept for 3 hours at 4°C, during which 6-MFA precipitated. The precipitate was dissolved in half-volume buffer saline adjusted to varying pH and solutions stored at −10°C overnight. This was followed by one cycle of thawing and centrifugation at 6,000 r.p.m. for 30 minutes. Supernatants were tested for antiviral activity. Results in Table 2 show that 6-MFA prepared in 2 batches and retained in phosphate buffer saline of pH 6 and 7 is more active (100 % mouse protection) than that prepared in pH 4, 5 and 8.

Table 1. Effect of varying the pH of the medium on the production and activity of 6-MFA

Fungal growth medium (pH)	6-MFA activity (2ml/mouse)	
	Survivors*	Survival (%)
2	0/8	0
4	1/8	13
6	8/8	100
7	3/8	38
8	1/5	20
Control (buffer saline)	0/10	0

* mice survived/total number

Storage at −10°C

6-MFA precipitated from different batches of crude filtrate by acetone, suspended in distilled water and their activity was tested.

Table 2. Activity of 6-MFA prepared and retained in buffer saline of different pH

pH	6-MFA activity (2 ml/mouse)			
	Batch 1		Batch 2	
	Survivors*	Survival (%)	Survivors	Survival (%)
4	4/8	50	4/10	40
5	4/8	50	5/10	50
6	6/8	75	10/10	100
7	6/8	75	10/10	100
8	3/8	37	5/10	50
Control (buffer saline pH 7.0)	0/8	0	0/10	0

* mice survived/total number; Batch 1 and Bach 2 were prepared at intervals of 8 weeks,

Table 3. Effect of storage (−10°C) of aqueous solution of 6-MFA

Batch No.	Activity of fresh 6-MFA				Days of storage	Activity of stored 6-MFA			
	Treated		Untreated control			Treated		Untreated control	
	Survivors*	Survival (%)	Survivors	Survival (%)		Survivors	Survival (%)	Survivors	Survival (%)
1	10/10	100	0/10	0	65 days	9/9	100	0/9	0
2	9/10	90	0/8	0	80 days	8/8	100	0/9	0
3	10/10	100	1/9	11	95 days	10/10	100	0/9	0
4	8/10	80	0/7	0	105 days	7/10	70	0/9	0
5	9/10	90	0/10	0	120 days	8/10	80	0/9	0
6	10/10	100	1/10	10	148 days	8/10	80	0/9	0

* mice survived/total number

After storage at −10°C they were taken out at intervals, thawed and again tested in mice. Data presented in Table 3 show that there was no appreciable loss of antiviral potency of 6-MFA on storage upto 148 days.

Thermostability

6-MFA samples prepared in distilled water and contained in Corning glass tubes (6″×1″), 20 ml in each, were exposed for 5 minutes to various temperatures, *viz.* 30°, 56° and 80°C in water bath and another at 100°C (boiling over flame). Still another was kept at 120°C (autoclave). All samples of 6-MFA were tested along with untreated control for activity in mice. Results (Table 4) show that when control mice (not receiving 6-MFA) showed almost 100 % specific mortality, treatment with 6-MFA, exposed to temperatures 30°, 56° and 80°C, protected 100 % of the test animals. However, with injection of 6-MFA heated to 100° and 120°C, no test mice could be protected.

Freeze-drying of 6-MFA from Different Solvents

6-MFA precipitated from crude filtrate was prepared as before. The precipitate was suspended in one-half volume of different solvents, *viz.* phosphate buffer saline (PBS), 4 % dextrose (w/v), glucose-nitrate-yeast broth and distilled water. The different solutions were kept at −10°C overnight, followed by one cycle of thawing and centrifugation as before. Supernatants of each were divided into two parts, one was stored at −10°C (control 6-MFA), and the other freeze-dried (test 6-MFA). Freeze-dried samples were reconstituted in distilled water prior to test in mice. Results (Table 5) of antiviral assay show that both the initial (before freeze-drying) and the final

Table 4. Effect of temperature on the stability of 6-MFA *in vitro*

6-MFA treatment		6-MFA activity (2 ml/mouse)			
		Batch 1		Batch 2	
		Survivors	Survival (%)	Survivors	Survival (%)
5 minutes at 30°C (room temperatuse)		10/10	100	8/8	100
Water bath	56°C	10/10	100	8/8	100
	80°C	10/10	100	8/8	100
	100°C	0/10	0	0/8	0
Autoclave	120°C	0/10	0	0/8	0
No 6-MFA (buffer saline)		0/10	10	0/8	0

* mice survived/total numcer; batch 1 and batch 2 were prepared at intervals of 8 weeks.

Table 5. Effect of freeze-drying on the stability of 6-MFA

Freeze-dried 6-MFA prepared from (solvents)	Initial (before freeze-drying)		Final (after freeze-drying)	
	Survivors*	Survival (%)	Survivors	Survival (%)
Buffer saline	10/10	100	10/10	100
Dextrose	8/8	100	10/10	100
Glucose-nitrate-yeast broth	8/8	100	9/9	100
Distilled water	9/9	100	8/10	80
No 6-MFA (control)	0/10	0	0/10	0

* mice surviving/total number

samples of 6-MFA (after freeze-drying), protected 80~100 % of the animals infected with a dose of virus that killed 100 % of the untreated control mice.

Dialyzability

Dialysis of the crude 6-MFA was done after reconstituting the freeze-dried sample with distilled water (1 mg/ml.). It was carried out in cellophane bags (Visking dialysis tubing) with an average pore diameter of 15~20 Å and exclusion limit M 10,000~20,000, at 4°C under stationary condition. The cellophane bags filled with 6-MFA were kept in beaker's containing triple distilled water and samples were removed at intervals of 2, 18 and 24 hours. These samples along with undialysed 6-MFA were tested for antiviral activity. Results (Table 6) show that 100 % of the test mice were protected with injection of 2 ml of either the dialysed or undialysed 6-MFA, while all infected mice in untreated control died within 8-day period. Undialysed 6-MFA (control) given at the rate of 0.25 ml per mouse, protected only 17 % of the test mice. Dialysis of this substance for 18 and 24 hours slightly increased its antiviral activity (33 %).

Filterability

Freeze-dried samples of 6-MFA was reconstituted (1 mg/ml) in distilled water and the aqueous solution filtered through 'Metricel' membrane filter (Gelman Instrument Co., U.S.A.) having pore size diameters of 0.2 and 0.45 u. Second sample was filtered through Millipore filter (Millipore Filter Corporation, Bedford, Mass., U.S.A). The third sample was passed through Seitz Ek pad. All these samples along with unfiltered 6-MFA (control) were tested for their activity. Results (Table 7) show that treatment with 1 ml of *unfiltered* 6-MFA protected 57 % ot the test mice under

Table 6. Effect of dialysis on the antiviral activity of 6-MFA

Treatment	Experiment I (0.25 ml/mouse)		Experiment II (2 ml/mouse)	
	Survivors*	Survival (%)	Survivors	Survival (%)
Dialysed for 2 hours	2/12	17	—	—
〃 〃 18 hours	4/12	33	12/12	100
〃 〃 24 hours	4/12	33	12/12	100
Undialysed 6-MFA (control)	2/12	17	12/12	100
Virus alone (control)	0/12	0	0/12	0

* mice survived/total number
— not done

Table 7. Effect of filtration on antiviral activity of 6-MFA

Filtration through	Experiment I (1 mg/mouse)		Experiment II (2 mg/mouse)	
	Survivors*	Survival (%)	Survivors	Survival (%)
'Metricel' membrane filter				
0.2 μ	5/8	62	10/10	100
0.45 μ	5/8	62	10/10	100
Millipore filter	5/8	62	—	—
Seitz Ek pad	4/7	57	—	—
Unfiltered (control)	4/7	57	10/10	100
Virus alone (control)	0/8	0	0/10	0

* mice survived/total number
— not done

Table 8. Effect of varying duration of ultracentrifugation on antiviral activity of 6-MFA

Supernatant 6-MFA samples taken out after centrifugation	Experiment I 6-MFA 2 mg/mouse (2 ml)		Experiment I 6-MFA 2 mg/mouse (1 ml)	
	Survivors*	Survival (%)	Survivors	Survival (%)
2 hours	4/8	50	—	—
4 hours	5/8	63	2/12	16
6 hours	5/8	63	—	—
8 hours	5/8	63	—	—
Uncentrifuged 6-MFA (control)	8/8	100	12/12	100
Virus alone (control)	0/8	0	0/8	0

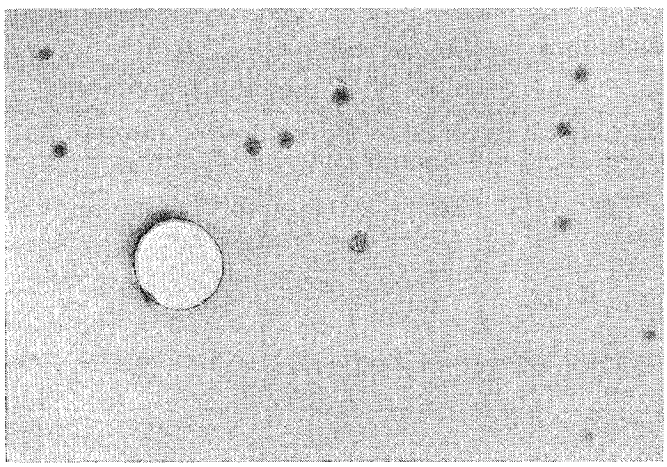
* mice survived/total number
— not done

the conditions when no infected mice survived in the untreated virus control. All samples of *filtered* 6-MFA injected under identical conditions also protected 57~62 % of the test mice (Exp. I). However, with 2 mg samples of filtered or unfiltered 6-MFA, 100 % of the test mice was protected (Exp. II).

Sedimentation

Freeze-dried sample of 6-MFA (2 mg/ml) were reconstituted in distilled water. Two experiments were performed. In one tubes, approximately 13 ml capacity, was filled with the 6-MFA solution and centrifuged at 40,000 r.p.m. (Beckman's Ultracentrifuge Model G) for varying intervals, *viz.* 2, 4, 6 and 8 hours. Supernatants were collected and tested along with uncentrifuged solution given at the rate of 4 mg per mouse (2 ml) for activity. Results (Expt. 1, Table 8) show that uncentrifuged solution of 6-MFA, serving as control, protected 100 % of the infected test mice, whereas all infected untreated animals died within 7~8 days. However, centrifugation of 6-MFA solution for 2, 4, 6 and 8 hours apparently reduced antiviral potency of the supernatants as these could protect only 50, 63, 63 and 63 % of the infected mice respectively.

Plate 1. Electron micrograph of 6-MFA showing virus-like particles (4×33,600)



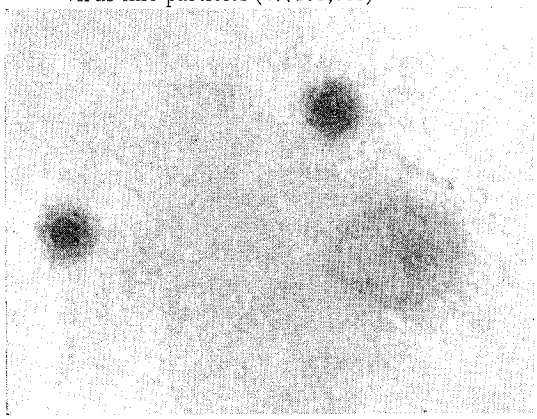
In another test, injection with uncentrifuged 6-MFA solution tested at the rate of 2 mg per mouse, *i.e.* 1 ml also protected 100 % of the infected mice. Ultracentrifugation of this sample at 40,000 r.p.m. for 4 hours reduced the antiviral activity of the supernatant (percentage of mice protected) to 16 %.

Electron-microscopy

Grids used for electron-microscopy were 200 mesh copper screen onto which a film of carbon had been deposited directly. Aqueous

solution of 6-MFA prepared at different times was negatively stained with 1 % uranyl acetate[7]. One drop of the sample was applied to a carbon-coated grid and washed off a minute later with two drops of distilled water. To the grid was added one drop of the stain followed by a second drop added a little later. After 30 seconds the stain was washed off with a drop of distilled water and the excess liquid blotted off with filter paper. Eight to ten grids were observed under Hitachi HV IIE-1 electron microscope with an accelerating voltage of 75 KV and photograph was taken on Fuji orthochromatic film.

Plate 2. Electron micrograph of 6-MFA showing virus-like particles (4×108,000)



Presence of virus-like particles was revealed, though in low concentrations (10~15 particles per microscpe field at magnification × 33,600). These were mostly pentagonal in shape with an average diameter of 300 Å (Fig. 1). The cores of the particles are deeply stained, and may contain nucleic acids and the outer coat (protein) consisting of capsomeres, somewhat more clear, can be seen (Fig. 2) under high power.

Discussion

6-MFA is fairly well tolerated (over 300 mg/kg) and its ED_{50} in mice is about 36 mg/kg, because 6-MFA has shown activity against human Chikungunya virus, preliminary investigations into its physico-chemical nature were undertaken.

6-MFA is apparently thermolabile, high molecular weight, nondialyzable, readily passing through filter of pore size diameter as small as 0.2 μ. Its high molecular weight is confirmed by test in centrifugation field. 6-MFA withstands freeze-drying and storage. Biosynthesis of 6-MFA in the mold *A. flavus* is affected by the pH of the growth medium, low and high pH being deleterious to its synthesis and slightly acidic medium (pH 6) favors it. 6-MFA is more stable *in vitro* at pH 6 and 7 in aqueous medium. It has been reported that polyhedral virus-like particles[8] are associated with helenine and statolon, the two antiviral agents obtained from *P. funiculosum* and *P. stoloniferum* respectively. 6-MFA in our case appears to contain virus-like particles pentagonal in shape with an average diameter of 300 Å. Yet we do not know the relationship between these virus-like particles and the antiviral activity in 6-MFA nor we know if the virus-like particles in 6-MFA is chemically or serologically related to the viruses in helenine or statolon, or to the recently reported viruses of *P. chrysogenum*, strains NRRL 1951, NRRL 1951. B-25, X-1612, Q-176, Wisc. 48-701, Wisc. 51-20 C and BRL 700[9], *P. cyaneofulvum*, strain CM I 58138[10], *A. niger*, strain IMI 146891 or *A. foetidus*, strain IMI 41871[11].

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