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

I. PRODUCTION AND ASSAY OF 6-MFA AGAINST SEMLIKI FOREST VIRUS INFECTION IN MICE

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6-MFA, a new antiviral agent isolated from *Aspergillus flavus*, strain 6-MFA, showed significant protection rates in mice against fatal Semliki Forest Virus (SFV) challenge. Major antiviral activity resided intracellularly in fungal mycelia. Maximum (100%) antiviral activity could be achieved after 5 days of incubation of the fungus under stationary conditions in SHOPE's medium. ED_{50} of crude 6-MFA is around 36 mg/kg body weight and the maximum tolerated dose, over 300 mg/kg body weight in mice. Mice protected by 6-MFA against initial SFV challenge remained refractory to subsequent challenge. No direct virucidal action of 6-MFA was observed.

MAHESHWARI *et al.*[1] and MAHESHWARI & GUPTA[2] reported earlier that *Aspergillus flavus*, strain 6-MFA produced an antiviral factor(s) designated 6-MFA in liquid cultures, effective against three viral infections, namely Semliki Forest, Chikungunya and neurovaccinia in mice. The production of 6-MFA in culture and its administration in mice to produce optimal antiviral effect is discussed in this paper.

Materials and Methods

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

Fungi *Aspergillus flavus*, strain 6-MFA was obtained as a clone from the parent culture of *A. flavus*, DU/KR/162 b, received from University of Delhi, India. *Penicillium funiculosum*, strain NRRL 2075 (producer of helenine), obtained as an agar slant from NRRL, Peoria, Ill., U.S.A., was used as a standard for comparison.

Virus The Semliki Forest Virus (SFV) of SMITHBURN and HADDOW[3] obtained from ATCC, U.S.A., was maintained in mice by intracranial inoculations at intervals of 3 months. The brains of the sick mice showing specific paralytic symptom were collected aseptically and homogenized in 10 % w/v HANKS BSS. This was then clarified by centrifugation at 3,000 r.p.m. for 30 minutes and supernatant stored in 1 ml aliquots and kept at −10°C. Dilutions of the virus were prepared in HANKS just prior to inoculation. The virus (0.5 ml) was inoculated subcutaneously (s.c.) into mice and LD_{50} was determined to be approximately $10^{-6.62}$ as calculated according to REED and MUENCH.[4]

Preparation of 6-MFA

One ml of an uniformly thick spore suspension (10^8/ml) prepared from a ten day old agar slant culture of *A. flavus* was inoculated into 1-litre conical flasks containing 200 ml of SHOPE's[5] broth. Flasks were incubated at 26°±1°C for 5~7 days under stationary conditions. Shake culture fermentation, carried out in a rotary shaker (Emenvee Engineers, Poona) at 250 r.p.m. for 72 hours

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at 26°±1°C, was also employed to see whether there was any significant differences in the 6-MFA production. After fermentation the mycelial mats (pellicle) were pooled and homogenized in total volume of the culture filtrate in a mixer (Mixi, Bajaj) for 3 minutes and the contents centrifuged for 30 minutes at 3,000 r.p.m. to remove mycelial debris. Chilled acetone was added in the ratio of 1 : 1 to the supernatant (crude filtrate) and the mixture was kept at 4°C for 3~4 hours for complete precipitation. The mixture was then centrifuged at 6,000 r.p.m. for 30 minutes. A sticky pale white pellet was sedimented. This was collected and then dissolved in sterile distilled water in one half the volume of the original crude filtrate and the solution stored at −10°C overnight and thawed at 37°C without agitation. The contents were then centrifuged at 6,000 r.p.m. for 30 minutes. The supernatant and pellet were tested for their antiviral activity. The supernatant (acetone fraction) containing the antiviral activity (designated 6-MFA) was kept at −10°C until further use and the pellet having little activity discarded. Freeze-drying of the supernatant yielded a pale white powder, and 1 ml of it yielded approximately 1 mg of dry crude 6-MFA. Approximately 1 kg of wet mycelia along with their culture filtrate (6 litres) yielded 3.0 g of crude 6-MFA.

Pellicle extracts were also prepared and tested for its antiviral activity; the extraction was done by homogenizing it with phosphate buffer saline in volumes equal to culture filtrate and supernatant was collected after centrifugation.

Crude helenine was prepared from *P. funiculosum* under conditions similar to that employed for the preparation of 6-MFA from *A. flavus*.

Antiviral assay

Unless stated otherwise samples of 6-MFA or helenine were injected before freeze-drying into mice by intraperitoneal (i.p.) route and virus challenge (100 LD_{50}) given 24 hours later by s.c. route.

Experimental

Activity of Fractions

The four fractions of *A. flavus* culture, namely, culture filtrate, pellicle extract, crude filtrate and acetone fraction, were tested and activity compared. One ml of each of these were injected and mice challenged by SFV, control mice receiving buffer saline. No death occurred due to toxic symptoms (non-specific death) within 24 hours. Onset of specific symptoms due to infection in the control mice occurred on the 4 th day after virus challenge. Results (Table 1) show that apparently little activity was associated with filtrate devoid of mycelia. Acetone precipitation tended to concentrate the antiviral activity.

Optimum Incubation Time

Fermentation was carried out under stationary condition in SHOPE's broth, and 6-MFA samples were prepared at different intervals (3~9 days). Mice were injected with aliquots of 2 ml each, and challenged later by SFV. Maximum protection (100 %) was obtained with 6-MFA prepared from 5 days old culture (Table 2).

Table 1. Comparison of the anti-Semliki Forest Virus activity of various fractions of *A. flavus*, strain 6-MFA in culture

Fractions	Experiment I		Experiment II	
	Survivors*	Survival (%)	Survivors	Survival (%)
Filtrate	0/9	0	1/8	12
Pellicle extract	4/9	44	3/9	33
Crude filtrate	3/9	33	3/9	33
Acetone fraction	4/8	50	5/9	55
Control (buffer saline)	0/8	0	0/8	0

* Mice surviving/total number

Shaken *versus* Stationary Fermentation

The effect of varying the conditions of fermentation on the production of 6-MFA or helenine in SHOPE's and in MOYER's broth[6] was studied. Mice were injected with 2-ml

aliquots of the two and challenged by SFV. 6-MFA and helenine prepared from cultures incubated in MOYER's broth under stationary condition protected 60 % and 20 % of mice, respectively. Under shake condition, the protection levels were 44 % and 36 %, respectively. However, when 6-MFA and helenine were isolated from cultures grown in SHOPE's medium, the protection levels of mice were 87 % and 34 % under stationary, and 70 % and 84 % under shake culture conditions, respectively (Table 3).

Table 2. Kinetics of production of 6-MFA by *A. flavus* in culture

6-MFA extracted after	Experiment I		Experiment II	
	Survivors*	Survival (%)	Survivors	Survival (%)
3 days	2/10	20	3/10	30
5 days	8/10	80	10/10	100
7 days	8/10	80	10/10	100
9 days	8/10	80	10/10	100
Control (buffer saline)	0/7	0	0/10	0

* Mice surviving/total number

Route of Administration of 6-MFA

Six experiments were performed in which dry 6-MFA dissolved in distilled water (2 mg/mouse) and virus were introduced by various routes. Hundred percent protection in mice

Table 3. Effect of varying the cultural conditions of fermentation on yield and potency of 6-MFA and helenine

Fermentation	Fungi	SHOPE's broth		MOYER's broth	
		Survivors*	Survival (%)	Survivors	Survival (%)
Stationary	*Aspergillus flavus* (6-MFA)	14/16	87	9/15	60
	Penicillium funiculosum (crude helenine)	6/18	34	3/15	20
	Control (buffer saline)	0/18	0	0/15	0
Shaken	*Aspergillus flavus* (6-MFA)	14/20	70	6/14	44
	Penicillium funiculosum (crude helenine)	16/19	84	5/14	36
	Control (buffer saline)	0/20	0	0/15	0

* Mice surviving/total number

Table 4. Effect of varying the route of administration on the antiviral activity of 6-MFA

Experiment	Route of 6-MFV	Route of SFV	Survivors+	Survival (%)
I	Intraperitoneal	Intraperitoneal	12/12	100
	Control (saline)	〃	0/12	0
II	Intraperitoneal	Subcutaneous	10/10	100
	Control (saline)	〃	0/10	0
III	Intravenous	Subcutaneous	7/7	100
	Control (saline)	〃	0/7	0
IV	Subcutaneous	Subcutaneous	8/10	80
	Control (saline)	〃	0/10	0
V	Oral	Subcutaneous	0/8	0
	Control (saline)	〃	0/9	0
VI	Intraperitoneal	Intracerebral	0/10	0*
	Control (saline)	〃	0/10	0

\+ mice surviving/total number
* symptom onset time lengthened by 48 hours.

was observed when SFV was given i.p. to mice administered 6-MFA by the same route. 6-MFA administered to mice i.p., intravenously (i.v.), s.c. or orally and challenged with SFV by s.c. route showed 100, 100, 80 and 0 % protection, respectively. However, no protection was seen when mice receiving 6-MFA i.p. were challenged with SFV intracranially (Table 4).

Time of Administration of 6-MFA

Two experiments were performed in which the quantity of 6-MFA administered was varied.

Table 5. Effect of varying the interval between 6-MFA administration and SFV challenge

Interval (Time in hours)	Experiment I		Experiment II	
	6-MFA injected prior to virus infection 2 mg/mouse		6-MFA injected after virus challenge 4 mg/mouse	
	Survivors*	Survival (%)	Survivors	Survival (%)
24	10/10	100	0/10	0
18	8/10	80	—	—
12	8/10	80	—	—
6	6/10	60	2/10	20
3	4/10	40	3/10	30
1	—	—	5/10	50
0	1/10	10	5/10	50
Virus alone (buffer saline)	0/10	0	0/10	0

* mice surviving/total number
— Not done

Table 6. ED_{50} of liquid 6-MFA

6-MFA (ml/mouse)	Mortality ratio	Died	Survived	Accumulated values			
				Died (D)	Survived (S)	Mortality ratio	Percent mortality $\frac{(D)}{(D+S)}\times 100$
0.1	7/8	7 ↑	1 ↓	22	1	22/23	95
0.2	6/8	6	2	15	3	15/18	83
0.5	5/8	5	3	9	6	9/15	60
1.0	4/8	4	4	4	10	4/14	28
2.0	0/10	0 *	10 *	0	20	0/20	0
2.5	0/8	0	8	0	28	0/28	0
3.0	0/8	0	8	0	36	0/36	0
3.5	0/10	0	10	0	46	0/46	0
4.0	0/10	0	10	0	56	0/56	0

* The arrows indicate the direction of addition for the accumulated values.

50% end point or the proportionate distance lies between 0.5 ml and 1.0 ml.

$$\text{Proportionate distance} = \frac{\text{mortality above } 50\% - 50}{\text{mortality above } 50\% - \text{mortality below } 50\%}$$

$$= \frac{60-50}{60-28} = \frac{10}{32} = 0.312$$

Negative log of the ED_{50} concentration = Negative log of dilution above 50 per cent mortality + Proportionate distance = 0.301 + 0.312 = 0.612

ED_{50} = 0.612 ml

Hundred percent protection of mice was observed if 6-MFA (2 mg/mouse) was administered 24 hours prior to infection with SFV. The protection rate however fell progressively when 6-MFA was administered 18, 12, 6, 3 and 0 hour before virus challenge to 80, 60, 40 and 10 % respectively (Table 5, Exp. I). If mice infected with SFV were administered 6-MFA (4 mg/mouse) at intervals of 0, 1, 3, 6 and 24 hours post infection percentage survival of 50, 50, 30, 20 and 0 respectively (Table 5, Exp. II) was obtained.

Minimum Effective Dose (ED_{50}) of 6-MFA

The ED_{50} of 6-MFA was determined using both liquid and freeze-dried samples. Mice were injected with varying concentrations of 6-MFA and challenged by SFV. From the cumulative percentage survival and cumulative percentage mortality, the 50 % end point was calculated according to REED & MUENCH. ED_{50} for liquid and freeze-dried 6-MFA was found to be 0.612 ml and 0.592 mg (approximately 36 mg/kg body weight) respectively (Tables 6 and 7).

In vitro Effect of 6-MFA on Virus

The *in vitro* effect of 6-MFA on virus was studied by preparing a mixture with known concentrations of 6-MFA and SFV dispensed in a number of tubes. The tubes were then incubated at 37°C. At intervals (every 30 minutes) contents of a tube were removed and injected i.p. into mice at the rate of 1 ml (2 mg 6-MFA+100 LD_{50} SFV) per mouse. Control mixture was prepared in buffer saline instead of 6-MFA in the same proportion. Results (Table 8) show that there is no change in the proportion of mice protected with increasing time of contact between virus and 6-MFA.

Table 7. ED_{50} of freeze-dried 6-MFA

6-MFA (mg/mouse)	Mortality ratio	Died		Survived		Accumulated values			
						Died (D)	Survived (S)	Mortality ratio	Percent mortality $\frac{(D)}{(D+S)}\times 100$
0.062	0/9	9	↑	0	↓	32	0	32/32	100
0.125	6/8	6		2		23	2	23/25	92
0.250	5/8	5		3		17	5	17/22	77
0.500	5/9	5		4		12	9	12/21	57
1.000	4/9	4	*	5	*	7	14	7/21	33
2.000	2/10	2		8		3	22	3/25	12
3.000	1/10	1		9		1	31	1/32	3
4.000	0/10	0		10		0	41	0/41	0
5.000	0/10	0		10		0	51	0/51	0

* The arrows indicate the direction of addition for the accumulated values.

50% end point or the proportionate distance lies between 0.500 mg and 1.000 mg.

$$\text{Proportionate distance} = \frac{\text{mortality above } 50\% - 50}{\text{mortality above } 50\% - \text{mortality below } 50\%}$$

$$= \frac{57-50}{57-33} = \frac{7}{24} = 0.291$$

Negative log of the ED_{50} concentration = Negative log of dilution above 50 per cent mortality + proportionate distance = 0.301 + 0.291 = 0.592

ED_{50} = 0.592 mg

Acquisition of Immunity in Mice Saved by Treatment with 6-MFA

Two experiments were performed. In the first, immunity test was performed with virus given by s.c. or i.c. route and results obtained with 6-MFA was compared with helenine. Three groups of mice (35 days old), one treated with 6-MFA (2 ml/mouse), another with helenine (2 ml/mouse), and third (control) which received buffer saline were challenged 24 hours later with 100 LD_{50} SFV by the s.c. route and animals observed for 14 days. Results (Table 8) show that 84 % of infected mice were protected by helenine treatment, and 70 % by 6-MFA, whereas none (col. 2) survived in the infected untreated control. On the 15 th day the survivors were rechallenged by SFV (100 LD_{50}) by s.c. or i.c. routes, and mice of similar age (50 days) were inoculated with SFV to serve as controls. All animals were observed for 14 days from the date of rechallenge. Data presented show that infected animals saved by 6-MFA as well as by helenine remained refractory to virus re-challenge, while a large proportion of the control mice (cols. 4 and 6) of similar age remained susceptible to SFV.

Table 8. Direct action of 6-MFA on SFV

Time of contact*	Survivors+	Survival (%)
0 minute	0/6	0
Control	0/6	0
30 minutes	0/6	0
Control	0/6	0
60 minutes	0/6	0
Control	0/6	0
90 minutes	0/6	0
Control	0/6	0
120 minutes	0/6	0
Control	0/6	0

* 6-MFA incubated with SFV for varying length of time at 37°C; control mixtures were incubated with buffer saline and virus.
\+ mice surviving/total number.

In the second experiment, similar immunity test was performed, but, with virus (100 LD_{50}) given through s.c. route only to mice saved by treatment with varying quantities (2.00~4.00 ml) of 6-MFA. Data (Table 10) show that a single treatment with 2.00 ml (approximately 3 ED_{50}) of 6-MFA was enough to protect and also to confer resistance in mice to further challenge.

Table 9. Susceptibility of mice saved by treatment with 6-MFA and helenine to SFV infection

Treatment (Acetone fractions)	Initial infection s.c.		Rechallenge s.c.		Rechallenge i.c.	
	Survivors* Col. 1	Survival (%) Col. 2	Survivors Col. 3	Survival (%) Col. 4	Survivors Col. 5	Survival (%) Col. 6
6-MFA	14/20	70	9/9	100	5/5	100
Helenine	16/19	84	8/8	100	7/8	88
Control (−) (buffer saline)	0/20	0				
Control (+) (buffer saline)			1/5	20		
Control (*) (buffer saline)					0/5	0

* mice surviving/total number
(−) 35 days old mice infected s.c.
(+) 50 days old mice infected s.c.
(*) 50 days old mice infected i.c.

Table 10. Susceptibility of mice saved by treatment with varying amounts of 6-MFA

6 MFA (Liquid acetone fraction)	Initial infection (s. c.)		Rechallenge (s. c.)	
	Survivors*	Survival (%)	Survivors	Survival (%)
2.0 ml	10/10	100	10/10	100
2.5 ml	8/8	100	8/8	100
3.0 ml	8/8	100	8/8	100
3.5 ml	10/10	100	10/10	100
4.0 ml	10/10	100	10/10	100
Control** (buffer saline)	0/10	0		
Control(*) (buffer saline)			4/12	33

* Mice surviving/total number
** 35 days old mice infected s.c.
(*) 50 days old mice infected s.c.

Discussion

Major antiviral activity of *Aspergillus flavus*, strain 6-MFA in culture was confined to the fungal pellicles. 6-MFA can be prepared by acetone fractionation of mycelial extract. Both stationary and shaken conditions of fermentation favoured production of anti-Semliki forest virus substance(s), however, reverse was the case with *P. funiculosum* NRRL 2075 (producer of helenine) where shake fermentation favoured helenine production. These results of ours with respect to helenine differ from that of SHOPE[7], who, by employing the stationary method of fermentation, obtained a product with 100% anti-SFV activity as against only 34% obtained by us. This difference may be attributed to several modifications we introduced in the production of crude helenine, namely, shortening of the incubation time (5~7 days instead of 6~10 days), and extracting helenine from the crude filtrate instead of extracting it from mycelial mat only with buffer saline as SHOPE did.

MOYER's (sucrose-nitrate-peptone) medium encouraged sporulation, while SHOPE's (glucose-nitrate-yeast) medium encouraged mycelial growth in *A. flavus*. Our experience indicated that SHOPE's medium is superior to MOYER's medium in the production of both 6-MFA and helenine.

Though 6-MFA has not been isolated in its pure state, yet it is interesting to note that even in its crude form it has significantly high protection rates in mice against SFV challenge. The ED_{50} of the crude 6-MFA is around 36 mg/kg body weight, and the maximum tolerated dose which is well over 300 mg/kg body weight suggests a wide margin of safety.

It is interesting to note that 6-MFA treated mice surviving initial infection of SFV remained refractory to further challenge, and so did the crude helenine treated mice. SHOPE[8] observed that infected mice saved by helenine treatment remain immune or susceptible to SFV depending upon the dose of virus used initially to infect the animals; with low doses of virus, mice saved by helenine succumbed to subsequent challenge. The mechanism of immunity in mice induced by 6-MFA treatment is under investigation.

The fact that 6-MFA is not directly virucidal (Table 8) and that time (24 hours) must elapse (Table 5) before 6-MFA treated animals develop full antiviral resistance, suggest that 6-MFA acted primarily on the host body. In our studies (accompanying paper) it is observed that suitable concentrations of cycloheximide, given 1 hour after 6-MFA administration at subeffective levels, produced significantly high rate of protection in mice against subsequent SFV challenge. Cycloheximide is known to enhance interferon production in cultured tissue cells pretreated with a variety of inducers[9~11]. It is likely that 6-MFA may be also inducing interferon in our mice. We have not attempted to isolate interferon from 6-MFA treated animals as yet, though there may be other mechanism(s) of host directed antiviral action of 6-MFA not involving interferon[12].

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