IN VITRO AND *IN VIVO* EVALUATION OF VERSICOLIN, AN ANTIFUNGAL ANTIBIOTIC

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Versicolin is mainly active against *Trichophyton rubrum*. It is inactivated by serum. However, attainable blood level can be fifteen to twenty times higher than the fungicidal concentration for as long as 4 hours after a single intravenous administration of the maximum tolerable dose of 25 mg/kg body weight of mouse. It has no haemolytic activity like other polypeptide antibiotics. Skin does not show the presence of any measurable amount of the antibiotic. It is excreted through urine to the extent of 65 % of the maximum tolerable dose of the antibiotic administered intravenously. Versicolin is effective against experimental infection by *T. rubrum* in guinea pigs at dose as low as 2.5 mg/kg body weight. The antibiotic shows no sign of subacute toxicity at these curative doses.

Only a few antifungal antibiotics produced by fungi have found application in curing superficial and systemic mycosis. In general antifungal antibiotics have *in vitro* activity against pathogenic fungi, but only a few have been reported to have *in vivo* activity against those causing superficial and systemic infections. However, clinical studies have been reported with amphotericin B,[1] hamycin[2] and nystatin.[3] The only orally active product is griseofulvin[4] which requires high dose and prolonged treatment.

Versicolin, a new antifungal antibiotic, was isolated from culture filtrate of *Aspergillus versicolor*[5,6,7] and a tentative structure has been reported.[8] The antibiotic is mainly active against *Trichophyton rubrum* which causes 90 % of the skin infections in eastern India. In the present paper, a study is reported on the biological properties of the antibiotic.

Materials and Methods

Determination of minimum inhibitory concentration in presence and in absence of serum

For determining the minimum inhibitory concentration (M.I.C.) of versicolin in absence of serum, SABOURAUD's broth (pH 6.4~7.0) containing 20 μg/ml each of penicillin G and streptomycin was used; for determining the MIC in presence of serum, double-strength SABOURAUD's broth (pH 6.4~7.0) was mixed with an equal volume of normal horse serum and the mixture preincubated for 2 hours at 37°C with penicillin G and streptomycin (20 μg/ml). Penicillin G and streptomycin were used to prevent contamination. Penicillin G and streptomycin did not affect the action of versicolin as predetermined by control experiments conducted without the addition of these antibiotics. The spore suspension (10^5 spores/ml, 5 ml) from a 3-week slant culture of each of the test organisms was inoculated into 5 ml of the above medium and incubated for 4 days at 30°C. For *Candida albicans*, 0.05 ml of the cell suspension (10^5 cells/ml) was used and the incubation time was 24 hours at 30°C. One-tenth ml of versicolin at various concentrations in 50 % ethanolic solution was added.

Acute toxicity of versicolin

Male albino mice weighing 18~22 g were used in these studies. A group of ten mice was

used for each of the doses being administered intravenously, intraperitoneally, subcutaneously or orally. Intravenous injections were made at a rate of 0.1 ml per second into the tail vein. The mortality was determined 72 hours after the administration of the antibiotic. The LD_{50} and 19/20 confidence limits were determined by the method of LITCHFIELD and WILCOXON[9] using six dosage levels.

Distribution in organs and blood

Groups of five male albino mice weighing about 20 g were given a single intravenous, subcutaneous or oral dose of 25 mg/kg, 70 mg/kg or 250 mg/kg body weight respectively. Collected blood and organs were pooled. Serum was separated and pooled organs were homogenized with an equal volume of buffered saline at pH 7.0. Antibiotic concentration of organs, serum as well as whole blood, was determined by a cup-plate method using *T. rubrum* as the test organism. For determining antibiotic activity in the skin[10] of animals, one cm^2 of the skin from the back of the test animal was stretched 1.5 times the original area and the epidermal layer scraped out by a blunt scalpel. The epidermal layer was suspended in buffered saline at pH 7.0, homogenized in a loosely fitting Potter-Elvehjem homogenizer and centrifuged. The supernatant was assayed for antibiotic activity by a cup-plate method using *T. rubrum* as the test organism.

Excretion of versicolin in urine and faeces

Groups of five albino Swiss male mice weighing about 20 g were given a single dose of versicolin by various routes, *viz.* intravenous, subcutaneous or oral. Urine and faeces were collected for 24 hours and antibiotic activity determined by a cup-plate method using *T. rubrum* as the test organism. Faeces were dissolved in 3 volumes of buffered saline and centrifuged, and the supernatant used for antibiotic assay as usual.

Protection of guinea pigs against dermatophytosis caused by *T. rubrum*

Experimental dermatophytosis of guinea pigs by *T. rubrum* was induced by the method of TAGAMI *et al.*[11] Guinea pigs weighing 300~400 g were used. The skin of the midback was used as the inoculation site. The spore suspension of *T. rubrum* (0.05 ml containing 200,000 spores) was delivered to the inoculation site. The site was immediately covered with a 2×2 cm sheet of polyethylene film and the inoculum spread by light pressure on the film, which was secured with an impermeable plastic tape and held in place for 72 hours by elastic adhesive bandage. The occlusive dressing was removed after 72 hours and the animals observed for 12~15 days. Versicolin in 0.5 ml of aqueous solution was given orally in daily doses of 25 mg/kg, 10 mg/kg, 5 mg/kg or 2.5 mg/kg to groups of three infected animals for 15 successive days and the animals observed for one month after the termination of treatment. Infected animals in the control group were kept under observation for the same period without antibiotic treatment. The presence or absence of the fungus in skin lesion of both the treated and untreated groups of animals was detected microscopically by direct smear method as well as by cultural isolation from hair and scales of the lesion.

Subacute toxicity of versicolin

The subacute toxicity of versicolin was tested in groups of four albino Swiss male mice 2~3 week-old, weighing about 20 g; these were given a daily oral dose of 25 mg/kg, 5 mg/kg or 2.5 mg/kg of versicolin in 0.5 ml of aqueous solution for 15 successive days. Control mice were given only water for the same period. Five days after the termination of administration, the animals were sacrificed to determine blood cell count and weights of different organs and also to make histological examination.

Results

Inhibitory Concentration in Presence and in Absence of Serum

Minimum inhibitory concentration of versicolin against different dermatophytes (Table 1) shows that the antibiotic is active against different species of *Trichophyton*, *Microsporum*, and against *Epidermophyton floccosum*, but it is mainly active (1.2~1.5 μg/ml) against *Trichophyton rubrum*.

Table 1. Minimum inhibitory concentration of versicolin in presence and in absence of serum

Test organisms	M.I.C. of versicolin (μg/ml)	
	In absence of serum	In presence of 50% serum
Trichophyton rubrum	1.2~1.5	24~25
T. mentagrophytes	28~30	ND
T. tonsurans	18~20	〃
Epidermophyton floccosum	14~16	〃
Microsporum canis	12~14	〃
M. gypseum	12~14	〃

ND: not determined

It may be pointed out that versicolin is inactive against *Candida albicans*, *Aspergillus flavus*, and *Aspergillus fumigatus* which cause systemic infections. The minimal inhibitory concentration in presence of serum against *T. rubrum* is 24~25 μg/ml, which shows that the antibiotic is greatly inactivated by serum.

Action of Versicolin on Red Blood Cell

The action of the antibiotic on red blood cell was determined by the method of P. DIMICK,[12] which shows that it has no haemolytic properties like other polypeptide antibiotics.

Acute Toxicity of Versicolin against Mice

The LD_{50} values (mg/kg body weight) of versicolin against mice are 33, 61, 80 and 330 (Table 2) for intravenous, intraperitoneal, subcutaneous and oral administration respectively.

The maximum tolerable dose (M.T.D.) which is the dose at which all the animals survived, and showed normal growth rate are 25, 40, 70 and 250 mg per kg body weight for intravenous, intraperitoneal, subcutaneous and oral administration respectively.

Table 2. Acute toxicity of versicolin in mice

Route of administration	M.T.D. mg/kg	LD_{50} mg/kg	Confidence limits mg/kg
Intravenous	25	33	30.4~35.8
Intraperitoneal	40	61	52.6~70.7
Subcutaneous	70	80	69.0~92.8
Oral	250	330	304~358

LD_{50} values were calculated by the method of LITCHFIELD and WILCOXON.[9]

Distribution of Versicolin in Blood, Serum and Organs

Groups of five animals were administered through the intravenous, subcutaneous or oral routes, doses of 25, 70 or 250 mg/kg body weight respectively; One hour after administration of the drug, the corresponding blood and serum levels of versicolin were 87~24, 40~18 or 54~18 μg/ml; these levels fell quickly to attain within 4 hours values of 24~18, 12~ trace or 12~ trace (Table 3). Generally the concentration of antibiotic in serum is much less than in whole blood, which indicates that a considerable portion of the antibiotic remains associated with cellular or noncellular elements of blood in the insoluble form. Studies on distribution of antibiotic in different organs (Table 4), such as liver, lung, spleen, kidney and skin indicate that

Table 3. Blood and serum level of versicolin after intravenous, subcutaneous and oral administration in mice

Doses (mg/kg body weight)		Concentration of versicolin (μg/ml)		
		1 hour	2 hours	4 hours
25 Intravenous	Blood	87	63	24
		87	60	18
	Serum	70	45	18
		24	18	trace
70 Subcutaneous	Blood	40	28	12
		40	28	12
	Serum	34	28	trace
		18	12	trace
250 Oral	Blood	54	40	12
		54	40	12
	Serum	50	38	trace
		18	12	trace

Groups of five albino mice weighing about 20 g were given a single intravenous, subcutaneous or oral doses of 25, 70 and 250 mg/kg body weight, respectively. Blood was collected at different intervals of time and antibiotic activity assayed by a cup-plate method using *T. rubrum* as the test organism.

Table 4. Distribution of versicolin in different organs of mice

Dose (mg/kg)	Organs	Concentration of versicolin (μg/mg)		
		1 hour	2 hours	4 hours
25 Intravenous	Liver	34	29	—
	Kidney	60	45	trace
	Lung	54	40	—
	Spleen	50	32	—
	Skin	—	—	—
70 Subcutaneous	Liver	24	18	—
	Kidney	50	40	trace
	Lung	—	—	—
	Spleen	—	—	—
	Skin	—	—	—
250 Oral	Liver	34	28	—
	Kidney	60	45	trace
	Lung	54	40	—
	Spleen	40	24	—
	Skin	—	—	—

—: Indicates absence of antibiotic activity.

Five albino mice weighing about 20 g were given a single intravenous, subcutaneous or oral dose of 25, 70, and 250 mg/kg body weight, respectively. Collected organs from five animals (killed at the time indicated) were homogenized with an equal volume of buffered saline at pH 7.0. The antibiotic activity was assayed by a cup-plate method.

the antibiotic is uniformly distributed except in skin where it did not reach a measurable concentration when administered by any of the three different routes. Kidney showed a little

preferential accumulation of the antibiotic whose concentration quickly diminishes till it becomes nil at 4 hours.

Excretion of Versicolin in Urine and Faeces

The drug is excreted in the active form, mostly in urine, and only slightly in faeces, although in case of intravenous administration excretion is exclusively through urine, the extent of excretion being 65% (Table 5) of the single maximum tolerable dose.

Table 5. Excretion of versicolin through urine and faeces

Route of administration	Dose (mg/kg)	% of versicolin excretion	
		Urine	Faeces
Intravenous	25	65	Nil
Subcutaneous	70	24	16
Oral	250	36	16

Groups of five albino mice each weighing about 20 g were given a single dose by different routes: intravenous, subcutaneous or oral. Urine and faeces were collected after 24 hours and antibiotic activity was assayed by cup-plate method using *T. rubrum* as a test organism. Faeces were dissolved (1:3) in buffered saline at pH 7.0 and centrifuged. The supernatant was used for antibiotic assay.

Table 6. Guinea pig protection test against experimental dermatophytosis by versicolin

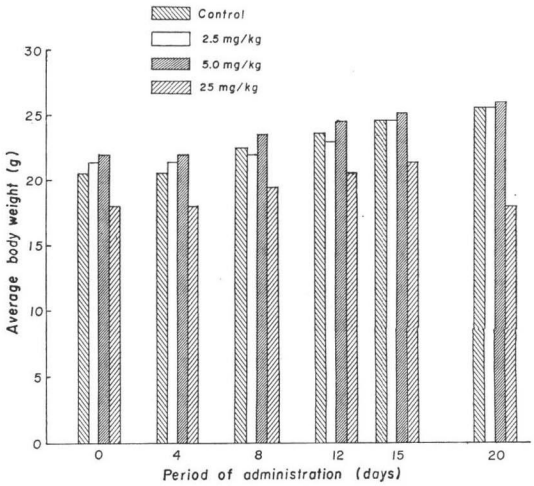
Dose (mg/kg body weight)	Initial body weight	Body weight after termination of dose	Days required for cure of infection	
			15	30
25 mg/kg×15 days	300	312	fully cured	—
	300	314	fully cured	—
	330	345	almost cured	—
10 mg/kg×15 days	270	282	fully cured	—
	305	315	fully cured	—
	250	270	fully cured	—
5 mg/kg×15 days	280	305	fully cured	—
	300	315	fully cured	—
	260	315	fully cured	—
2.5 mg/kg×15 days	240	270	Some spots on the midback of the skin during 15 days treatment	fully cured
	280	320		fully cured
	260	300		fully cured
Without dose (Infected control)	280	325	Vigorous infection	Vigorous infection
	280	305		
	290	305		

Five groups each containing 3 guinea pigs, experimentally infected (b.w. 250~350 g) were given a daily dose (mg/kg b.w.) of 25, 10, 5, 2.5 or 0 (control) orally for 15 days. Then the animals were kept for one month for observation by direct smears as well as by cultural isolation of *T. rubrum*. After 15 days of drug administration 3 groups showed complete cure but the group receiving 2.5 mg/kg b.w. of drug required another 15 days more to be fully cured.

Protection of Guinea Pigs from Experimental Dermatophytosis with *T. rubrum*

The doses used were 2.5, 5, 10 and 25 mg/kg which were equivalent to 1/100, 1/50, 1/25 and 1/10 of the M.T.D. obtained from acute toxicity studies. The effect of versicolin was tested at different oral doses in guinea pigs which were experimentally infected with *T. rubrum* (Table 6). All the infected animals were cured at dose as low as 2.5 mg/kg×15. The lower dose required about one month, whereas the higher doses (25 mg/kg×15 or 5 mg/kg×15) needed only 15 days. The untreated animals in the control, observed for one month, showed vigorous infection.

Fig. 1. Effect of oral administration of different doses of versicolin in young mice



Subacute Toxicity of Versicolin

Versicolin was found to show no subacute toxicity upon administration of 15 daily doses of 25, 5 or 2.5 mg/kg body weight to young male mice. All the animals showed similar gain of body weight (Fig. 1). No death was observed during the period of observation. Five days after the termination of adminis-

Table 7. Subacute toxicity of versicolin

Dose (mg/kg b.w.) ×15 days	Body weight		Blood cell count		Average weight of organs (4)					
	Initial (g)	Termination of treatment (g)	WBC	RBC 1×10^4	Liver	Spleen	Kidney	Brain	Lung	Heart
2.5	25	27	8,950	850	1.120	0.105	0.396	0.300	0.172	0.125
	22	25	9,000	825	1.199	0.099	0.310	0.280	0.168	0.124
	19	23	7,800	650	1.100	0.092	0.240	0.270	0.150	0.108
	18	21	—	—	—	—	—	—	—	—
5	22	25.5	9,000	1,010	0.950	0.083	0.309	0.260	0.153	0.107
	22	25.5	9,560	950	1.100	0.088	0.340	0.272	0.162	0.115
	24	26.0	10,100	1,000	1.210	0.100	0.370	0.288	0.160	0.121
	19	22.0	—	—	—	—	—	—	—	—
25	19	22	10,110	960	0.893	0.088	0.245	0.245	0.142	0.098
	19	24	10,100	960	1.120	0.107	0.365	0.285	0.163	0.121
	19	24	9,920	815	1.110	0.107	0.365	0.280	0.165	0.122
	17	20	—	—	0.810	—	0.205	0.245	—	—
Control	22	26	11,100	1,040	1.290	0.109	0.378	0.289	0.169	0.138
	21	25	9,550	925	1.210	0.100	0.372	0.269	0.160	0.134
	20	25	9,310	780	1.110	0.100	0.372	0.265	0.160	0.123
	19	24	9,640	760	1.110	0.089	0.370	0.265	0.157	0.122

Four groups each of 4 Swiss albino mice were administered a daily dose of 25, 5, 2.5 or 0 mg/kg b.w. (control) orally for 15 days. Five days after the termination of treatment the mice were sacrificed and blood cell counts, weight of different organs were made and compared with the control. There was no noticeable change in the parameters observed.

tration, all the animals were killed and their blood and organs collected for examination. As shown in Table 7, red and white blood cell counts and also weights of different organs were within the normal range. Microscopic and histological examination did not indicate any abnormal change in liver and kidney tissues.

Discussion

It appears that the antibiotic is active against different species of dermatophytes, and is mainly active against *T. rubrum*, the inhibitory concentration being 1.2 μg/ml. It is inactivated by serum.

Studies on acute toxicity indicate that the LD_{50} by oral route is 330 mg/kg body weight as against 33 mg/kg body weight by intravenous route. Thus the oral toxicity of the antibiotic is, like hamycin,[13] fairly low. It is of interest to note that the maximum tolerable dose, for example by intravenous route, is 25 mg/kg body weight, which seems to be fairly high when compared to the antifungal dose *in vitro*. It was therefore worthwhile persuing the *in vivo* distribution of the antibiotic in blood and different organs under different routes of administration. Studies on distribution indicate that blood can maintain 15~20 times the fungicidal concentration for as long as four hours after intravenous administration of a single maximum tolerable dose of the antibiotic.

Skin did not attain measurable concentration after any route of administration, as the method employed for its assay was very crude. It is of interest to note that on the basis of studies by GENTLES *et al.*,[14] in which one g of hair from a guinea pig fed griseofulvin, an active drug, for three weeks, yielded only 5~6 μg, it would not be expected under the condition of the present experiment that a measurable concentration of the drug might be achieved in infected structures of the skin.

Excretion studies showed that the drug has no cumulative effect due to its excretion through urine to the extent of 65 % of the single maximum tolerable dose given by intravenous route.

It appears from protection test that versicolin is effective against experimental infection by *T. rubrum* in guinea pigs at dose as low as 2.5 mg/kg body weight when administered daily for 15 successive days by an oral route. The lowest dose afforded cure in one month, while the highest dose (5 mg/kg body weight) in only 15 days. It is of interest to note that versicolin gives complete protection against dermatophytosis, although it could not be detected in the skin. This might be taken to mean that the method cannot measure fungicidal concentration *i.e.* 1.2 μg per ml even if attained in the skin.

It is gratifying to note that the antibiotic showed no sign of subacute toxicity at these curative doses, as revealed by growth rate, blood picture, weight of different organs and microscopic and histological examinations.

In conclusion versicolin may be an orally useful antifungal antibiotic.

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