J. Biosci., Vol. 16, Numbers 4, December 1991, pp 243-250. © Printed in India.

Effect of griseofulvin on lipid composition and membrane integrity in *Microsporum gypseum*

INDU BALA CHUGH, M P GUPTA and G K KHULLER*
Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

MS received 16 April 1991; revised 16 September 1991

Abstract. The effect of griseofulvin on lipid constituents and membrane permeability of *Microsporum gypseum* has been investigated. Mycelia grown in medium containing griseofulvin (IC_{50} concentration) possessed a lower content of total lipids, phospholipids and sterols. This inhibitory effect was further supported by decreased incorporation of [^{14}C] acetate in total lipids, total phospholipids and sterols. Decrease in total phospholipids was also reflected to a varying extent in all individual phospholipids. An increase in the unsaturated to saturated fatty acid ratio was observed in mycelia grown in medium containing griseofulvin. Membrane permeability was affected by griseofulvin as shown by increased K^+-efflux and greater leakage of intracellular [^{32}P] labelled components from prelabelled cells. Our results suggest that the antifungal activity of griseofulvin is partially due to its secondary effect on lipid constituents of *Microsporum gypseum.*

Keywords. *Microsporum gypseum;* griseofulvin; phospholipids; membrane integrity.

1. Introduction

Griseofulvin is an orally effective antimicrobial agent for superficial fungal infections of the skin (Shah 1980). It is thought to be essentially fungistatic rather than fungicidal (Kerridge 1986) and its fungistatic action has been suggested to be due to interference with the synthesis of cell wall chitin (Blank *et al* 1960). This view was later rejected due to a lack of correlation between alterations in chitin level and the growth inhibitory action of griseofulvin. McNall (1960) observed a partial reversal of griseofulvin action by purines, pyrimidines and other nucleotides, thus suggesting the fungistatic action through direct interference with the synthesis of nucleic acids. However, Weinstein and Blank (1960) reported that griseofulvin is bound to lipids within the cell but not to RNA or DNA. Although the growth inhibitory activity of a number of antimycotics has been shown to be mediated through their interactions with lipid constituents (Kerridge 1986), no study has been initiated to examine the effect of griseofulvin on lipid constituents in dermatophytes. In the present paper, we have studied alterations caused by griseofulvin in lipid composition of *Microsporum gypseum.* As lipids are important membrane components, further experiments were conducted to examine the effect of the drug on membrane permeability and activity of membrane-bound enzymes.

2. Materials and methods

2.1 *Materials*

Griseofulvin and adenosine 3', 5'-cyclic monophosphate were procured from Sigma

*Corresponding author.

Chemical Co., St. Louis, Mo, USA. *Naja naja* snake venom was obtained from V P Chest Institute, New Delhi. Aminonaphtholsulphonic acid and trichloroacetic acid, 2, 5-diphenyloxazole (PPO) and 1, 4-bis (5-phenyloxazolylbenzene) (PPOP) were purchased from Sisco Research Laboratories Pvt. Ltd., Bombay and peptone was purchased from Centron Research Laboratories, Bombay. [^{14}C] acetate (sp. activity, 60.3 mCi/mmol) was obtained from the Bhabha Atomic Research Centre, Bombay.

2.2 *Organism and growth conditions*

M. gypseum (NCPF 412) obtained from the Mycological Reference Laboratory, School of Medicine, London, was maintained on Sabouraud's dextrose-agar slants (pH 5.4–5.6) at 27°C. The culture was regrown in liquid Sabouraud's medium (pH 5.4-5.6) containing 4% glucose and 1% peptone on a shaker (60 RPM)at27°C. To determine IC_{50} and minimum inhibitory concentration (MIC) doses of griseofulvin, cultures were grown in the presence of the drug at concentrations ranging from 0·1 to 0·8 μg/ml. The drug was dissolved in ethanol (0·2 mg/ml) and the concentration of the solvent in growth medium did not exceed 0·1 %, at which fungal growth or lipid constituents remain unaffected (Bansal and Khuller 1981). IC_{50} and MIC doses were derived from the graph prepared by plotting dry weight of mycelia at mid log phase (96 h old culture) versus concentration of griseofulvin in the medium.

2.3 *Lipid composition*

Effect of griseofulvin in growing mycelia was studied by growing *M. gypseum* in Sabouraud's broth containing IC_{50} dose of the drug (0·21 μg/ml). Control cultures were grown in the same medium without drug. Cells were harvested at mid-log phase (after 96 h) to analyse various lipid constituents. Lipids were extracted from mycelia by the method of Folch *et al* (1957) and were quantitated gravimetrically. Total phospholipids (TPL) were estimated by measuring inorganic phosphorus content in lipid extracts according to the method of Marinetti (1962). Sterols were extracted by the method of Singh *et al* (1979) and measured by the method of Zlatkis *et al* (1953).

2.4 *Identification of lipid components*

Individual phospholipids were separated on silica gel H plates by using chloroform: methanol: ammonia (65:25:4, v/v) solvent system as described by Kates (1972). Individual phospholipid components were localized by staining with iodine vapours and quantitated by measuring lipid phosphorus. To assay phospholipid fatty acids, these were converted to methyl esters by transesterification with methanol and thionyl chloride according to the modified method of Prabhudesai (1978) and analysed on 5700 AIMIL Nucon gas Chromatograph by using a column containing 20% diethylene glycol succinate (DEGS) on 60–80 mesh Chromosorb W. Fatty acids were identified by comparing their retention time with those of standards. Relative fatty acid composition was calculated by triangulation of peak areas.

2.5 *Incorporation of* [^{14}C] *acetate in lipids of M. gypseum*

The effect of griseofulvin on lipid biosynthesis in mid-log phase mycelia of M. *gypseum* was studied by the method of Khuller *et al* (1984). Mid-log phase mycelia were harvested and suspended in sterile 10 mM citrate phosphate buffer (pH 6·5) containing MIC dose (0·5 μg/ml) of griseofulvin and preincubated on a shaker at 27°C for 3 h. To study the biosynthesis of total lipids (TL), TPL and sterols, mycelia were further incubated with [^{14}C] acetate (1·5 μCi/g/15ml) at 27°C for 0 and 120 min. After incubation with [^{14}C] acetate, mycelia were separated, washed to remove adsorbed radioactivity and subjected to lipid extraction (Folch *et al* 1957) or sterol extraction (Singh *et al* 1979). Phospholipids were separated from neutral lipids by thin-layer chromatography of TL using acetone as the solvent. Radioactivity in TL, TPL and sterols fractions was determined using toluene based scintillation fluid.

2.6 *Measurement of* K^+*-efflux*

Membrane permeability of drug-treated mycelia was monitored by studying K^+-efflux. Mid log phase untreated mycelia were transferred into 10 mM citrate phosphate buffer (pH 6·5). Total intracellular K^+ content and the level of K^+ in filtrate was measured on a Na^+/K^+ Analyser 4020 (Orion Research, USA). Griseofulvin was then added to the cell suspension at 15 X MIC (7·5 μg/ml) and 30 X MIC (15 μg/ml) doses and percentage stimulation in K^+-efflux was measured after 15, 30, 45 and 60 min of incubation with the drug. The amount of K^+ release was expressed as a percentage of total intracellular K^+ as described by Chen *et al* (1977).

2.7 *Leakage of* [^{32}*P*] *labelled material from prelabelled cells*

Mid-log phase mycelia were harvested, transferred to fresh medium containing [^{32}P] labelled orthophosphoric acid (0·5 mCi/g cells in 100 ml growth medium) and incubated on a shaker at 27°C for 3 h. Mycelia were then isolated, washed thoroughly with chilled isotonic saline and transferred again into fresh medium containing griseofulvin at a dose of 10 X MIC (5 μg/ml) or only carrier solvent (ethanol 0·5%, v/v). Aliquots of the mycelial suspension were removed after 0, 1, 2, 3 and 4 h of incubation with the drug and filtrate was collected and used for monitoring the leakage of intracellular [^{32}P] by measuring radioactivity. Percentage release of intracellular [^{32}P] was calculated as described earlier (Gupta *et al* 1991).

2.8 *Assay of membrane bound enzymes*

Mid-log phase mycelia were harvested, and incubated in fresh sterile Sabouraud's medium containing 10 × MIC of griseofulvin (5 μg/ml) for 3 h at 27°C. Mycelia were separated again, washed with isotonic saline and resuspended (approximately 1 g cell/5 ml) in 40 mM Tris HCl buffer (pH 7·5) and disrupted by sonicalion in a sonifier (cell Disruptor, Model 195) for 5 min at 55 watts with cooling after each minute of sonication to keep the samples cold. The homogenate was subjected to

Table 1. Lipid composition of *M. gypseum* cells grown in the presence and absence of griseofulvin (IC_{50}).

Parameter	Control cultures (mg/g dry weight)	Griseofulvin grown (mg/g dry weight)
Total lipids	86·14 ± 10·23	58·88 ± 2·57**
Total phospholipids	16·24 ± 0·13	9·39 ± 1·5**
Total sterols	14·60 ± 0·05	9·10 ± 1·6**
Lysophosphatidylcholine	1·91 ± 0·05	1·26 ± 0·33**
Phosphatidylserine + Phosphatidylinositol	3·53 ± 0·80	1·94 ± 0·19*
Phosphatidylcholine (PC)	6·17 ± 0·70	4·12 ± 0·32**
Phosphatidylethanolamine (PE)	2·88 ± 0·27	0·97 ± 0·21***
Unknown phospholipids	1·70 ± 0·11	1·00 ± 0·34*
PC/PE ratio	2·14	4·24

Values are mean ± SD of three independent batches analysed in duplicate. *P<0·05; **P<0·01; ***P<0·001; IC_{50}–0·21 μg/ml.

cold centrifugation at 4°C at 10,000 *g* for 30 min. Post 10,000 *g* supernatant was taken in a dialysis bag (exclusion limit 6,000 daltons) and dialysed against 20 mM Tris HCl (pH 7·5) buffer for 7–8 h at 4°C. The dialysate was used to assay the activity of phosphodiesterase (PDE) by the method of Aboud and Burger (1971) and activity of 5'-nucleotidase was measured by the method of Heppel and Hilmoe (1951).

3. Results

3.1 *Lipid composition of control and griseofulvin-grown mycelia*

Table 1 shows the lipid composition of control and griseofulvin grown (IC_{50}) mycelia. Content of TL, TPL and sterols were found to decrease significantly (P <0·01) by 32, 42 and 38%, respectively in griseofulvin grown mycelia. Analysis of individual phospholipid fractions of control and griseofulvin grown mycelia is also shown (table 1). A significant decrease in the level of lysophosphatidylcholine (P<0·01), phosphatidylserine and phosphatidylinositol (P<0·05), phosphatidylcholine (P<0·01), phosphatidylethanolamine (P<0·001) and unidentified phospholipids (P<0·05) was seen in griseofulvin grown mycelia. The ratio of PC/PE increased from 2·14 to 4·24 in griseofulvin grown cells as compared to control.

The relative percentage of fatty acids in phospholipids from control and griseofulvin grown cultures have been shown in table 2. Griseofulvin grown mycelia showed a considerable decrease in myristic acid (32%) and palmitic acid (16%) with an increase in stearic acid content. However the levels of palmitoleic acid and oleic acids did not change significantly. The ratio of unsaturated to saturated fatty acids was 1·5 in control while it was 1·8 in the griseofulvin grown mycelia.

3.2 *Effect of griseofulvin on lipid biosynthesis*

Figure 1 shows lipid biosynthesis from [^{14}C] acetate in control and drug treated (0·5 μg/ml) mycelia. Uptake at 0 min was 10–20 times less than the 120 min value

Table 2. Fatty acid composition of phospholipids of control and griseofulvin grown (IC_{50}) *M. gypseum.*

	Relative percentage of phospholipid fatty acids	
Fatty acids	Control	Griseofulvin grown
Myristic acid (14:0)	6·82	4·61
Palmitic acid (16:0)	24·60	20·20
Palmitoleic acid (16:1)	3·66	3·97
Stearic acid (18:0)	8·57	10·66
Oleic acid (18:1)	18·82	18·87
Linoleic acid (18:2)	37·56	41·66
Unsaturated/saturated fatty acid ratio	1·5	1·8

Values are mean of two independent observations. IC_{50} — 0·21 μg/ml.

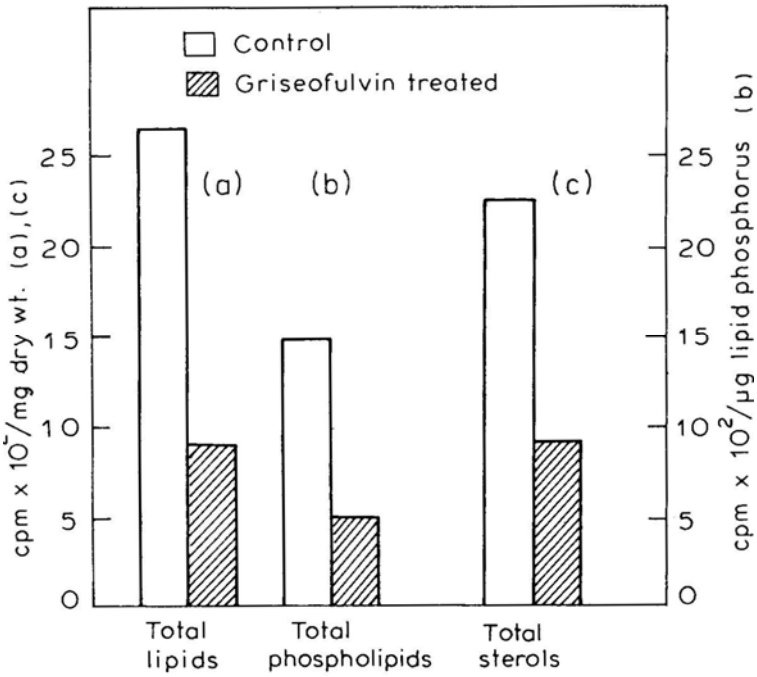

Figure 1. Effect of minimum inhibitory concentration of griseofulvin (0·5 μg/ml) on incorporation of [^{14}C] acetate into TL, TPL and total sterols of *M. gypseum* mycelia

and therefore the values represented in figure 1 are derived after subtracting 0 min value. Incorporation of [^{14}C] acetate in TL, TPL and sterol fractions was decreased by 33·9, 33 and 39·9% respectively due to the presence of griseofulvin.

3.3 *Effect of griseofulvin on leakage of cellular constituents*

Griseofulvin treatment (10 X MIC) of mid log phase cells resulted in a significant stimulation in leakage of intracellular [^{32}P] from prelabelled cells (174% in griseofulvin treated cells in comparison to 82% in control cells) after 4h of treatment with drug (table 3).

3.4 *Effect of griseofulvin on K^+-efflux*

Griseofulvin at 15 X MIC and 30 X MIC doses caused a stimulation in K^+-efflux by 18 and 28% respectively (figure 2) after 60 min of incubation with the drug. There was no eflux of K^+ for 60 min in the absence of the drug,

Table 3. Effect of griseofulvin on leakage of radioactivity from control and griseofulvin treated (10 X MIC) mycelia previously labelled with [^{32}P] (H_3PO_4).

	Percentage increase in [^{32}P] leakage	
Incubation time (h)	Control	Griseofulvin treated
1	37·5 (3)	71·9 (3)
2	84·8 (3)	137·3 (3)
3	87·6 (3)	160·9 (3)
4	81·7 (3)	174·3 (3)

Values in parenthesis represent number of independent batches analysed in duplicate. Experiment was started with same number of counts in control and griseofulvin treated cultures.

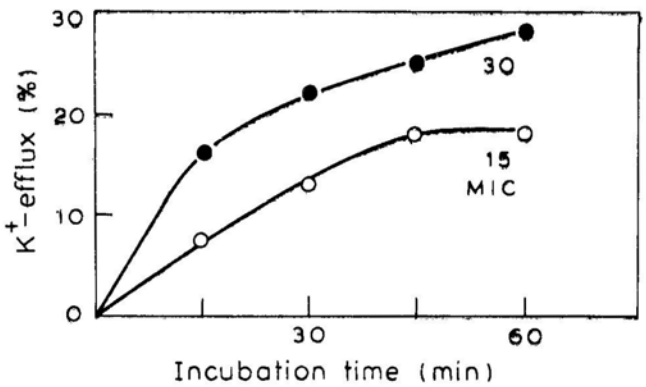

Figure 2. Effect of griseofulvin at 15 X MIC and 30 X MIC on K^+-efflux in *M. gypseum* cells. Values are average of three batches analysed in duplicate (O), 15 X MIC; (=) , 30 X MIC.

3.5 *Effect of griseofulvin on membrane-bound enzymes*

Activity of phosphodiesterase was not affected by griseofulvin. The enzyme activities were 24·6±3·0 and 28·0±4·0 nmol/min/mg protein in control and griseofulvin treated (10 X MIC) mycelia, respectively. There was no effect of griseofulvin on the activity of 5'-nucleotidase which was 10·0 ± 1·9 nmol/min/mg protein in control.

4. Discussion

Biochemical events leading to the growth inhibitory action of griseofulvin are yet not clear except for its inhibitory effects on chitin (Brian 1960) and nucleic acid synthesis (McNall 1960). In the present study, cells grown in the presence of IC_{50} dose of griseofulvin exhibited significantly lower levels of TL, TPL and sterols (table 1). This is probably due to the effect of the drug or its metabolites on the enzymes of lipid biosynthesis/degradation or through some alternate mechanism. However, experimental evidence is required to negate this hypothesis.

Further, an increase in K^+-efflux was observed from the mycelia treated with high doses of griseofulvin (15 X MIC and 30 X MIC; figure 2). This observation is supported by earlier reports by Iwata *et al* (1973) according to which high concentrations of clotrimazole caused leakage of various small molecules like K^+, amino acids, sugars and inorganic phosphates. Increased K^+ -efflux caused by clotrimazole (Iwata *et al* 1973) is known to decrease the cellular pH which has been proposed to activate certain lytic enzymes leading to enhanced degradation of some cellular constituents (Lampen 1966). In the present study, the observed increase in leakage of [^{32}P] components from griseofulvin treated cells (table 3) can be attributed to these facts. Antifungal drugs (e.g., polyenes and imidazoles) are also known to influence certain membrane-bound enzymes including phosphodiesterase and 5'-nucleotidase in *Candida albicans* (Surarit and Shephard 1987). However, griseofulvin did not alter the activity of these enzymes in *M. gypseum.*

On the basis of results presented here, it is clear that griseofulvin exerts its fungistatic effect through alterations in lipid composition and membrane permeability, in addition to the previously reported inhibitory effect on nucleic acid synthesis.

Acknowledgement

The work was financed by research grant to GKK from Indian Council of Medical Research, New Delhi.

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