INHIBITION BY GRISEOFULVIN OF MICROTUBULE ASSEMBLY IN VITRO

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1. Introduction

The antimitotic drug, griseofulvin, has been shown to arrest cell division at a point close to metaphase in a variety of cells [1]. The microtubules in griseofulvin-blocked HeLa cells appear to have a normal morphology and orientation within the spindle [2] and it has been suggested that griseofulvin inhibits some aspect of microtubule function rather than microtubule assembly [3]. Using a crude extract of brain, Wilson and Bryan [1] were unable to inhibit in vitro microtubule assembly, as measured by electron microscopy, with griseofulvin at a concentration known to inhibit mitosis (60 μM).

We report here that griseofulvin at concentration between 20 and 200 μM does inhibit in vitro assembly of purified microtubule protein from brain as measured by turbidimetry, electron microscopy and a sedimentation method. Inhibition of assembly by griseofulvin is distinctly different from inhibition by colchicine. Disc gel electrophoretic analysis indicates that microtubule protein polymerized in the presence of griseofulvin is depleted in the high molecular weight (HMW) components which normally co-purify with tubulin [4,5].

2. Experimental

Microtubule protein was purified from fresh sheep brains by two cycles of assembly in the presence of 4 M glycerol at 37°C and disassembly at 0°C [6]. Pellets of twice polymerized protein were stored in solid CO_2 without deterioration for at least 1 month. Immediately before use, the pelleted material was resuspended in cold glycerol-free assembly buffer (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP, pH 6.9), depolymerized at 0°C for 30 min and spun at 130 000 *g* for 30 min at 4°C to remove aggregated protein. The supernatant, stored on ice, was used for assembly experiments in the subsequent 4–5 h during which time there was no decrease in ability to form microtubules.

The kinetics of microtubule assembly at 37°C were monitored by turbidimetry [7] at 400 nm in a Gilford recording spectrophotometer. The increase in light-scattering at 400 nm correlated well with the appearance of microtubules detected by electron microscopy. Stock solutions of griseofulvin and colchicine in DMF were added such that the final concentration of DMF was 2% (v/v). This concentration of organic solvent did not affect the extent of polymerization or the morphology of microtubules formed, although the rate of polymerization was somewhat faster than in the absence of DMF. Polymerization was initiated by warming the sample from 0 to 37°C.

Samples for electron microscopy were prepared by negative staining [4]. SDS gel electrophoresis was according to the method of Laemmli on 7.5% acrylamide gels. Protein was determined by the method of Lowry [9].

3. Results and discussion

Fig.1 shows that griseofulvin inhibited both the rate and extent of polymerization of microtubule

Abbreviations: PIPES, piperazine-*N,N'*-bis (2-ethane-sulphonic acid); EGTA, ethylene glycol-bis-(β-aminoethylether) tetra-acetic acid; DMF, dimethyl formamide; SDS, sodium dodecyl sulphate.

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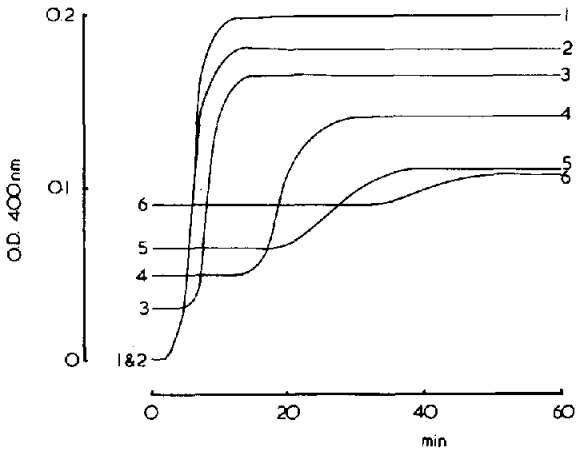

Fig.1. Inhibition by griseofulvin of microtubule assembly assayed by turbidimetry. Polymerization of microtubule protein at 2 mg protein/ml in assembly buffer, containing 2% (v/v) DMF, was initiated by warming from 0 to 37°C. Griseofulvin concentrations were: (curve 1) 0 and 5 μM; (curve 2) 20 μM; (curve 3) 50 μM; (curve 4) 80 μM; (curve 5) 100 μM; (curve 6) 200 μM.

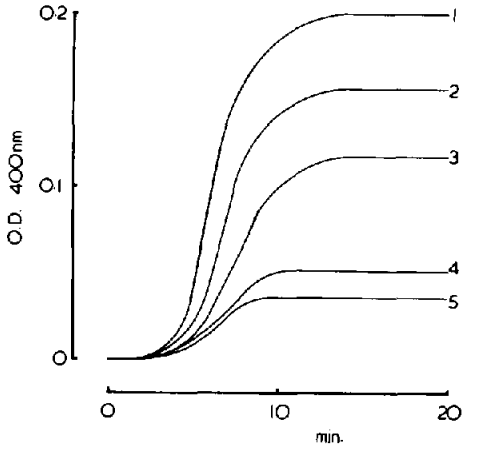

Fig.2. Inhibition by colchicine of microtubule assembly at 37°C. Microtubule protein was at 2 mg/ml in assembly buffer containing 2% (v/v) DMF. Colchicine concentrations were: (curve 1) 0; (curve 2) 1 μM; (curve 3) 3 μM; (curve 4) 5 μM; (curve 5) 10 μM.

protein. Addition of griseofulvin to a solution of microtubule protein at 0°C caused a very rapid increase in turbidity, hence the increase in initial absorbance values in fig.1. No microtubules were detected in samples taken at this point. Inhibition of microtubule formation was confirmed by electron microscopy and by sedimentation of polymerized material at 130 000 *g* for 30 min at 26°C, followed by protein estimation of pellet and supernatant fractions (table 1). 50–60% of the material polymerized in the presence of 100 μM griseofulvin could not be disassembled at 4°C and is probably the protein aggregated at 0°C on inhibitor addition.

Griseofulvin, but not colchicine (fig.2), caused a marked increase in the lag phase which precedes polymerization. A progressive increase in lag phase was also observed when the concentration of microtubule protein was lowered in the absence of inhibitor (fig.3). Pure tubulin dimer (at 2 mg protein/ml) prepared by ion exchange chromatography of microtubule protein, shows a lag phase of some 20 min before a very slow

Table 1

Recovery of microtubule protein incubated in assembly buffer with or without 100 μM griseofulvin for 60 min at 37°C, then spun at 130 000 *g* for 30 min at 26°C to yield H pellet and H supernatant. H pellets, resuspended in assembly buffer, were dialysed against 100 vol assembly buffer for 18 h at 4°C, then spun at 130 000 *g* for 30 min at 4°C to yield C pellet and C supernatant

	Percentage recovery of protein	
	Control	Griseofulvin-treated
Recovery of starting material	90.8	90.8
H pellet	64.3	29.0
H supernatant	26.5	61.0
Recovery of dialysed material	88.9	89.0
C pellet	16.9	53.2
C supernatant	72.0	35.8

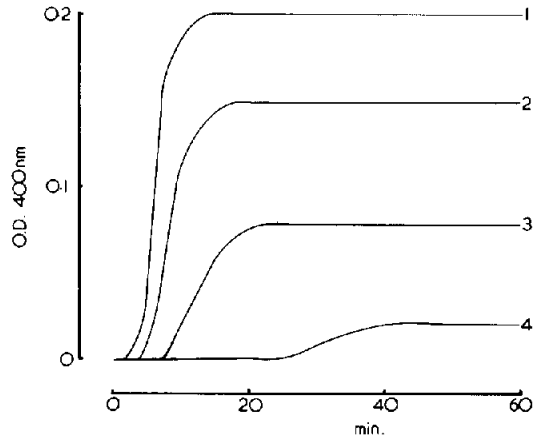

Fig.3. Effect of protein concentration on microtubule assembly at 37°C. Microtubule protein in assembly buffer containing 2% (v/v) DMF was at the following concentrations: (curve 1) 2 mg/ml; (curve 2) 1.6 mg/ml; (curve 3) 1.1 mg/ml; (curve 4) 0.6 mg/ml.

polymerization starts [5]. These data suggested to us that the increase in lag phase was due to depletion by dilution or chromatography of microtubule associated proteins and that griseofulvin, but not colchicine prevented the normal association of these proteins with the microtubule, thus causing an increase in lag phase. Colchicine is known to bind specifically to tubulin dimer [10].

Table 2 shows an SDS gel electrophoretic analysis of supernatants from microtubule protein which had been polymerized with or without griseofulvin, then spun at 130 000 *g* for 30 min at 26°C. The control supernatant was depleted in the HMW bands whereas the supernatant from griseofulvin treated material was enriched in HMW content relative to the starting material. The depletion in HMW content of the control supernatant is probably caused by the absence of glycerol in the incubation medium. Microtubule protein polymerized under these conditions contains 15–20% [4] HMW material against 4–12% HMW content of microtubules formed in glycerol [11].

Griseofulvin (100 μM) did not inhibit polymerization in assembly buffer containing 4 M glycerol. Lee and Timasheff [12] have shown that purified tubulin dimer at 2 mg protein/ml polymerises rapidly in the presence of glycerol with a lag phase of only 2 min. These data suggest that, in the presence of glycerol, the HMW components are not essential for rapid polymerization and consequently polymerization is insensitive to griseofulvin.

The failure to detect inhibition of microtubule assembly in vitro by griseofulvin reported previously [1] could be due to either the use of crude brain extract or the method of assay. We observed some microtubules by electron microscopy in microtubule protein polymerized in the presence of griseofulvin, although there were fewer than in control material. The turbidimetric and sedimentation assays, however, clearly demonstrated that the extent of polymerization was markedly inhibited by griseofulvin. If, as our data indicate, griseofulvin inhibits the association of HMW material with the microtubule, the microtubules observed after in vivo treatment with griseofulvin [2] may be lacking in components essential to their normal function in cell division.

Table 2

Effect of griseofulvin on the distribution of microtubule protein between HMW and tubulin dimer fractions separated by SDS gel electrophoresis. Microtubule protein was incubated in assembly buffer with or without 100 μM griseofulvin for 60 min at 37°C, then spun at 130 000 *g* for 30 min at 26°C to yield H supernatant. Aliquots containing 20 μg protein were then subjected to SDS gel electrophoresis. Destained gels were scanned at 650 nm in a Gilford model 240 spectrophotometer equipped with linear transport and the peak areas determined by integration.

	% Total protein		Ratio HMW
	HMW	dimer	dimer
Starting material	8.5	86.9	0.098
Control H supernatant	2.3	89.8	0.026
Griseofulvin-treated H supernatant	12.5	84.3	0.148

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Additional note

During preparation of this paper, inhibition by griseofulvin of in vitro assembly of pig brain microtubules has been reported by Weber et al. (J. mol. biol. (1976) 102, 817–829).

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