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GRISEOFULVIN AT LOW CONCENTRATION INHIBITS THE RATE OF MICROTUBULE POLYMERIZATION IN VITRO[1]

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SUMMARY: Effects of griseofulvin at concentrations of 0.1 mM or greater may be artifacts of supersaturation or non-specific protein aggregation. More biologically relevant effects have been sought at low concentrations, and inhibition of microtubule polymerization and depolymerization rates have been found (apparent K_i = 12.5 μM). Both forward and reverse rates are affected similarly, and there is little effect on the in vitro polymerization equilibrium at these concentrations.

Griseofulvin is an antibiotic product of Penicillium griseofulvum that has been shown to block mitosis (1,2). Although the effect seen in the light microscope is similar to the action of colchicine (3), biochemical comparisons show distinct differences in the action of the two drugs (4). Biochemical studies of the mode of action of griseofulvin have produced many contradictory results, with some reports that microtubules are disrupted by the drug (5-7) while others have failed to detect effects on microtubule polymerization (8). Griseofulvin has been shown to bind specifically to the microtubule-associated proteins (9), but also to inhibit the polymerization of tubulin in the absence of microtubule-associated proteins (7). The limited solubility of griseofulvin in aqueous solutions may account for this anomalous behavior. It has been common practice to dissolve griseofulvin in a water-miscible solvent such as dimethylformamide (5) or dimethylsulfoxide (6). Solubilization in aqueous buffer can be achieved by rapid dispersal of a concentrated organic solution of griseofulvin. The solubility limit for griseofulvin in water is 30 μM (1).

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In much of the work reported above, this has been exceeded. The result is either a supersaturated solution, if the dispersal has been effective, or a solution of much lower concentration than intended if aggregation has occurred. It has been suggested that griseofulvin binds non-specifically to the hydrophobic regions of proteins (9). With a supersaturated solution of the drug, it is possible that such non-specific effects go beyond mere binding, and protein inactivation or aggregation may thus be non-specific also.

Effects on microtubules in tissue culture cells can be observed at concentrations of 10 μM or less, where griseofulvin is fully soluble (6). To avoid possible artifacts of supersaturation, I have investigated the action of low concentrations of griseofulvin on microtubule polymerization in vitro, and have demonstrated inhibition of the rates of microtubule polymerization and depolymerization.

MATERIALS AND METHODS

Bovine brains were obtained courtesy of the abattoir, Dept. of Animal and Poultry Science, University of Guelph. Buffers, GTP (product No. G5881) and griseofulvin were from Sigma Chemical Company, St. Louis.

Microtubule protein was prepared by a modification (10) of the method of Murphy and Hiebsch (11). Purification was carried through three cycles of assembly and disassembly. The buffers used contained 0.1 M morpholinoethanesulfonic, pH 6.6, 1 mM $MgCl_2$, 1 mM GTP and 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid. Protein concentration was measured by the method of Lowry (12), relative to a bovine serum albumin standard. A sample colorimetrically equivalent to 1.0 mg/ml serum albumin contained 9.3 μM tubulin (13).

Polymerization measurements were made as described (10, 13), except that polymerization was induced by temperature step up from 8° to 30° and depolymerization by a return to 8°. Thermostatically jacketed cuvets were used, and sample volume was limited to 0.5 ml to achieve rapid temperature change (within 0.5° of steady state in 45 s). A brief vacuum pre-treatment to degas samples prevented bubble formation on cuvet windows during warm-up.

Griseofulvin solutions at concentrations up to 50 μM were made without organic solvent by suspending an excess of griseofulvin in aqueous buffer. The suspension was stirred vigorously at 60° for 15 minutes, decanted to remove solid, and cooled to 25°. The final concentration was determined and adjusted as necessary by measurement of UV absorbance at 296 nm (εmax 23442). For more concentrated solutions, a stock solution of 20 mM griseofulvin in dimethylsulfoxide was dispersed in the aqueous sample. Dispersal to obtain a supersaturated solution was most successful when griseofulvin was the final addition to the mixture and required agitation to mix without delay.

RESULTS AND DISCUSSION

Griseofulvin is relatively insoluble in aqueous solution. It has been common practice to prepare concentrated solutions in organic solvents such as dimethylsulfoxide and to disperse a small portion in aqueous buffer to obtain an aqueous solution. The inclusion of 2% dimethylsulfoxide in the aqueous solution did not greatly increase the solubility of griseofulvin (Fig. 1), although supersaturated solutions up to 0.2 mM in both pure aqueous and aqueous-2% dimethylsulfoxide buffers persisted without precipitation for several days. Evidence for the completeness of dispersal was obtained from adherence to Beer's Law and by fluorescence measurements. As aggregation proceeded, absorbance declined and fluorescence became quenched. Inclusion of 1% bovine serum albumin increased overall solubility dramatically, although it is unlikely that the true concentration of free griseofulvin exceeded the normal limit. The binding of griseofulvin by serum albumin probably resulted from the function of this protein as a carrier of small hydrophobic molecules. This observation may be of great significance for studies invovling cells in culture, since mammalian cell culture media frequently contain serum albumin.

Supersaturated solutions of griseofulvin cause aggregation of microtubule protein (7,14). The hydrophobic nature of microtubule proteins may make them more susceptible, but my results suggest that this phenomenon may be non-specific. Interactions with non-microtubule proteins have been observed. In an attempt to measure solubility in the presence of microtubule-depleted bovine brain supernatant protein, excess griseofulvin at 27° caused the solution to become highly turbid in a few hours, and absorbance measurements were not possible. Also, an arbitarily selected, normally stable enzyme, fumarase, was tested in the presence of griseofulvin. Fumarase activity was partly lost in 100 μM griseofulvin, but not in 67 μM. The loss of activity increased with time of exposure at 25°, and was not reversible on dilution (Table 1). This behavior suggested that highly supersaturated griseofulvin inactivated the enzyme rather than acting as a typical reversible inhibitor.

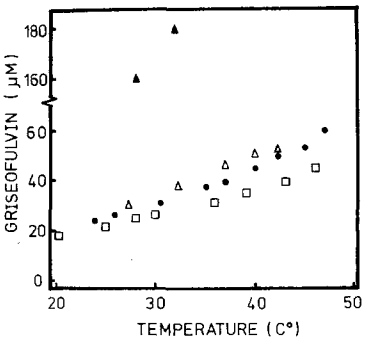


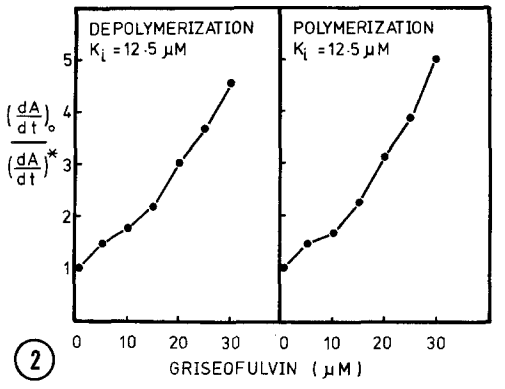
Figure 1. Solubility of griseofulvin in aqueous solution. An excess of griseofulvin crystals was stirred at the temperatures indicated. Concentration was determined from absorbance measurements at 296 nm, several consecutive readings being made, to ensure that the solution was in equilibrium with solid. Solutions: ● , distilled H_2O; □ , 0.1 M morpholinoethanesulfonic acid buffer, pH 6.6; △ , 2% (v/v) dimethylsulfoxide in buffer as above; ▲ , 1% (w/v) bovine serum albumin in buffer as above.

Microtubule polymerization was tested in the presence of griseofulvin. In preliminary experiments, effects were seen only at high concentration, and confirmed the work of others (5,6). The effects observable at lowest concentration were on the rate of cold-induced depolymerization. Cold-induced depolymerization is rapid, has no lag, and accurate measurement of this rate is difficult (15). A rapid temperature change is required, or the kinetics of depolymerization would be masked by the kinetics of cooling.

TABLE 1

Inhibition of fumarase by griseofulvin

Treatment	V_{max} (mol min^{-1} mg^{-1} protein)
No inhibitor	350
67 μM griseofulvin	342
100 μM griseofulvin:- added with substrate	201
added 10 min before substrate	126
added 10 min before substrate, diluted to 50 μM griseofulvin when substrate added	156



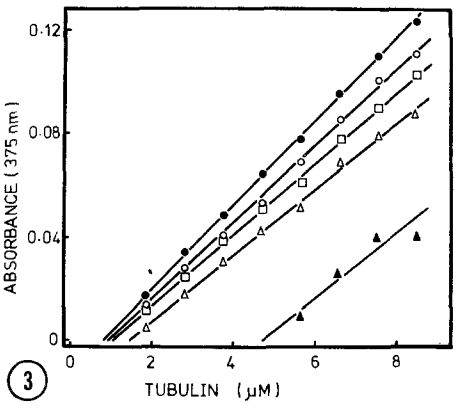


Figure 2. Inhibition of depolymerization and polymerization rates in the presence of griseofulvin. Microtubules (9.3 μM tubulin) were polymerized in the presence of griseofulvin, and maintained at 30° for 40 minutes A temperature step-down to 8° induced depolymerization, and the initial linear rate was measured. After 15 minutes at 8°, the temperature was returned to 30° and the initial polymerization rate was measured. The mechanistic significance of these rates has been explained by Johnson and Borisy (16). The graphs above are plotted in terms of the ratio of rate in the absence of inhibitor $(dA/dt)_o$ to the rate in the presence of inhibitor $(dA/dt)^*$. The concentrations shown are for total griseofulvin, and free griseofulvin has not been estimated.

Figure 3. Effect of griseofulvin on microtubule polymerization equilibrium, based on measurement of critical concentration. Polymerization of microtubules was measured spectrophotometrically: the change in turbidity at 375 nm, reversible on cooling to 0°, is recorded above. Critical concentration is the intercept on the concentration axis. Griseofulvin concentrations: ● , zero; o , 20 μM; □ , 40 μM; Δ , 60 μM; ▲ , 80 μM; no polymerization observed for 100 μM.

The procedure was refined by a combination of fast cooling ($t_{1/2}$= 7 s) and a reduction in the actual rate of depolymerization by use of a jump from 30° to 8° rather than 37° to 0°. Under these conditions, the inhibition of depolymerization rate was found to be nearly proportional to griseofulvin concentration up to the solubility limit of 30 μM (Fig. 2).

The effect of griseofulvin on microtubule polymerization equilibrium was tested by measurement of critical concentration. Critical concentration is the reciprocal of the polymerization equilibrium constant (16). Little effect was seen except at high concentration (Fig. 3). Above 40 μM griseofulvin, the turbidity increase due to polymerization was no longer fully reversible on cooling, suggesting some aggregation phenomenon other than normal microtubule polymerization contributed to the turbidity.

TABLE 2

Dependence of inhibition by griseofulvin on duration of pre-exposure.

Pre-exposure conditions	Inhibition, $(\frac{dA}{dt})_o / (\frac{dA}{dt})^*$:-	
	Depolymerization rate	Polymerization rate
0 min, 30°	-	1.2
10 min, 30°	1.3	2.0
20 min, 30°	1.6	2.2
30 min, 30°	2.6	2.6
40 min, 30°	3.0	3.1
30 min, 0°	-	1.3

Griseofulvin concentration was 20 μM and microtubule protein 9.3 μM with respect to tubulin. Rates were determined as described in Fig. 2. The condition shown as 0 min, 30° refers to the initial polymerization.

Since the polymerization equilibrium was unchanged at low drug concentration, both polymerization and depolymerization rates should be affected. No significant effect on polymerization rates was observed in the standard assay, until griseofulvin concentrations exceeded 40 μM. In this assay, polymerization was observed at the start, and depolymerization some 30 minutes later. The effect of duration of exposure to griseofulvin on depolymerization rates was then tested. This was found to be a critical factor determining the degree of inhibition (Table 2). Preincubation with the drug in the cold had little effect on the inhibition, and 40 minutes at 30° was necessary to see maximum inhibition. Polymerization rates measured after 40 minutes pre-exposure to griseofulvin at 30° showed inhibition comparable to depolymerization rates (Fig. 2). It should be noted that the depolymerization rate shown is the cold-induced rate at 8° and not the steady state rate at 30°.

Electron microscopy of microtubules polymerized in the presence of griseofulvin up to 40 μM showed normal morphology. Length distibutions were broader, with the appearance of a large subpopulation <1 μm not found in the absence of drug. Microtubule number concentration is therefore not decreased,

and the rate decrease must reflect changes in rate constants when inhibitor is present.

In conclusion, it should be noted that significant effects on rates of microtubule polymerization and depolymerization have been observed with low concentrations of griseofulvin. The effects escape notice under routine conditions for the measurement of microtubule polymerization, and previous investigators may have raised concentrations of griseofulvin to excessive levels in order to force a response from the system. Since experiments *in vivo* or cells cultured *in vitro* are unlikely to experience such artificially high concentrations of the drug, I feel that the low concentration effects described here are more likely to be biologically relevant. In particular, because inhibition of rate has been observed without a corresponding shift in equilibrium, my results would be consistent with a prevention of microtubule function *in vivo* without necessarily a disruption of microtubule structure.

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