EVIDENCE THAT GRISEOFULVIN BINDS TO A MICROTUBULE ASSOCIATED PROTEIN

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1. Introduction

We have recently shown that the anti-mitotic drug, griseofulvin, inhibits assembly of brain microtubules in vitro [1]. Similar data have been presented by Weber et al. [2]. Our earlier experiments indicated that griseofulvin inhibits microtubule assembly by preventing the association between tubulin and the microtubule associated proteins (MAPs) which normally co-purify with tubulin [3] and promote assembly of purified tubulin dimer [4].

We now present evidence that griseofulvin binds specifically to MAPs and not to tubulin dimer at concentrations of griseofulvin which inhibits microtubule assembly.

2. Experimental

Microtubule protein was prepared from fresh sheep brain and stored as described previously [1,5]. Immediately prior to use, twice-polymerised microtubule protein was resuspended in a small volume of cold PEMG buffer (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, pH 6.9) depolymerised at 0°C for 30 min and spun at 130 000 × *g* for 30 min at 4°C. Free and loosely bound glycerol was removed from the supernatant (CS2) by passage through a 1 × 40 cm column of G-25 Sephadex equilibrated with PEMG buffer at 4°C.

Tubulin dimer and MAPs were separated by chromatography on DEAE–Sephadex A-50 at 4°C. CS2 Protein, 100 mg, was applied to a 2 × 16 cm column of A-50 DEAE–Sephadex equilibrated with PEMG buffer and the column washed with 1 bed vol. of the same buffer, prior to elution with a 300 ml linear salt gradient 0–0.8 M KCl, in PEMG buffer at a flow rate of 12 ml/h. The absorbance of the column effluent at 280 nm was monitored in a Spectro-plus (MSE Ltd, Crawley, Sussex, England) flow cell and 4 ml fractions collected. The elution profile of such a column is shown in fig.1.

Although the absorbance at 280 nm of pooled fractions in peak 1 was 0.570, the protein concentration was less than 0.15 mg/ml indicating that the absorbance at 280 nm in this peak was largely due to material other than protein. The peak 1 pool was concentrated by ultrafiltration through an Amicon

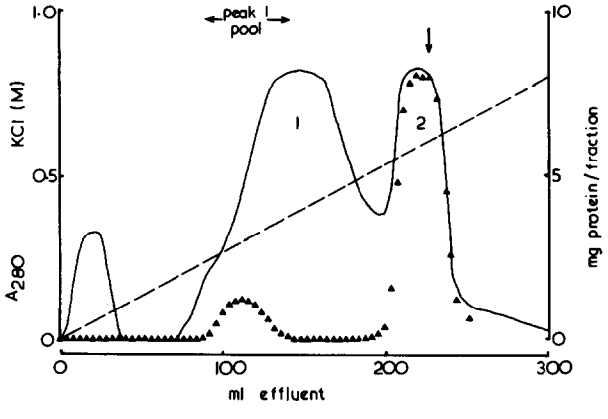

Fig.1. Separation of MAPs and tubulin dimer by chromatography on DEAE–Sephadex A-50. Experimental details are given in the text. A_{280} (———), mg protein/fraction (▲), KCl (M) (– – – –).

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

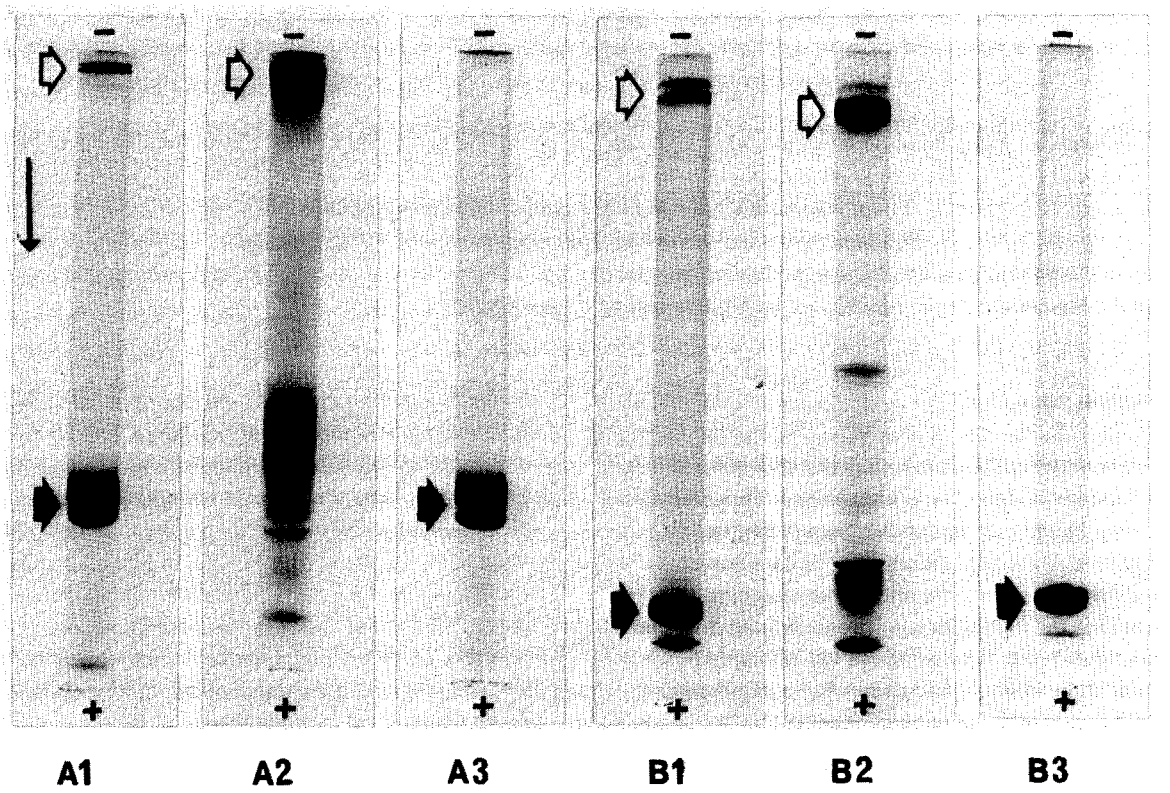

Plate 1. SDS–gel electrophoresis of microtubule proteins separated by chromatography on DEAE–Sephadex. (A) 7.5% Acrylamide, (B) 5% acrylamide. (1) 25 μg unfractionated microtubule protein, (2) 50 μg peak 1 pool protein, (3) 25 μg peak 2 fraction protein. (▷). MAPs, (◗) tubulin dimer, (→) direction of electrophoresis.

UM10 membrane to a final vol. of 5 ml. The protein concentration in fractions from peak 2 (60–70% loaded protein) was such that a single fraction provided adequate material for experimentation. The concentrated peak 1 pool and a fraction from peak 2 (indicated by the arrow in fig.1) were desalted on a 1 × 40 cm G-25 Sephadex column equilibrated with PEMG buffer and used immediately. Binding studies were completed within 30 h of resuspending the microtubule protein. Plate 1 shows an SDS electrophoretic analysis of peak 1 and peak 2 components. Peak 1 was essentially microtubule protein minus tubulin dimer and contained high molecular weight proteins, Tau protein [6] and several other minor components which were not identified. Peak 2 was tubulin dimer with a trace of high molecular weight protein.

Randomly tritiated griseofulvin was prepared at the Radiochemical Centre, Amersham, England and purified by thin-layer chromatography on silica (Polygram Sil G/ultraviolet, 254 nm, Camlab Ltd, Cambridge, England) with chloroform–acetone (93:7) [7]. Material chromatographing with standard griseofulvin was eluted with DMF and was found to be homogeneous during further chromatography and mass spectrometric analysis. Purified [^{3}H]griseofulvin (225 Ci/mol) was stored as a 4.4 mM solution in DMF at −30°C. Dilutions of this stock in unlabelled griseofulvin in DMF were prepared as required.

We initially attempted to measure griseofulvin binding to microtubule protein under equilibrium conditions using established techniques, namely, equilibrium dialysis, equilibrium gel-filtration [8] and rapid ultrafiltration through an Amicon 'Centriflo'

membrane (Type CF 25). Each of these methods proved unsuitable for binding studies with griseofulvin. At concentrations in excess of saturated aqueous concentration (~ 35 μM) griseofulvin in 2% DMF precipitated in the presence of dialysis membrane and the gel-filtration media used (G 25 Sephadex and Bio-Gel P-6). Binding studies under equilibrium conditions were therefore restricted to griseofulvin concentrations below 35 μM.

Griseofulvin inhibits microtubule assembly at concentrations from 20–200 μM [1]. Griseofulvin binding within this concentration range was measured by a non-equilibrium procedure in which free and loosely-bound griseofulvin was adsorbed by charcoal. Figure 2 (a) and 2 (b) show that incubation for 10 min at 0°C with 4 mg/ml charcoal (Norit-GSX, Norit-Clydesdale Co. Ltd, Glasgow, Scotland) followed by centrifugation at 2000 × *g* for 5 min at 4°C removed > 99% free griseofulvin from solution. Figure 2 (c) shows that griseofulvin binding to microtubule protein at 0°C was rapid and plateaued within 10 min of griseofulvin addition.

The routine binding assay procedure was as follows: 'T' samples, containing 500 μl protein in PEMG buffer (0.5–2.5 mg protein/ml) + 10 μl [^{3}H]griseofulvin in DMF (final concentration 5–150 μM in 2% DMF) were incubated for 10 min at 0°C before addition of 100 μl stirred charcoal suspension (final concentration 4 mg charcoal/ml). After mixing, samples were left for 10 min at 0°C, then centrifuged at 2000 × *g* for 5 min at 4°C. Aliquots, 0.1 ml, of supernatant were mixed with 1 ml PCS solubilizer (Hopkin and Williams Ltd, Chadwell Heath, Essex, England) and radioactivity determined in a Packard

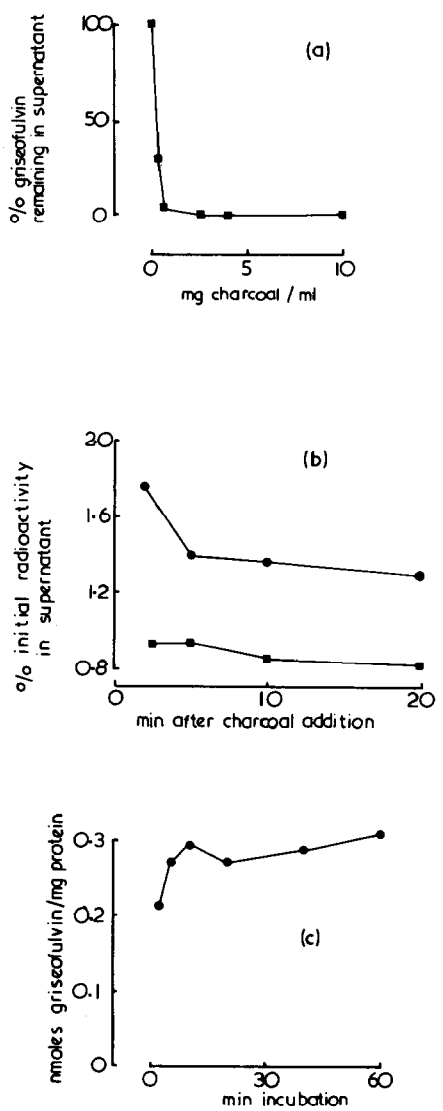

Fig.2. Measurement of griseofulvin binding to microtubule proteins using charcoal to adsorb free and loosely bound griseofulvin.

(a) Griseofulvin adsorption by different charcoal concentrations. PEMG buffer, 500 μl, containing 100 μM griseofulvin in 2% DMF was incubated with 100 μl water (100% control) or charcoal suspension to give the final charcoal concentrations indicated for 10 min at 0°C. After centrifugation at 2000 × *g* and 4°C for 5 min radioactivity in 0.1 ml aliquots of supernatant was determined.

(b) Time course of griseofulvin adsorption by charcoal. PEMG buffer, 500 μl, containing 100 μM griseofulvin in 2% DMF (▪) and 500 μl PEMG buffer containing 2 mg microtubule protein/ml and 100 μM griseofulvin in 2% DMF (•) were incubated for 10 min at 0°C before addition of 100 μl charcoal suspension (24 mg/ml). Samples were then incubated for the indicated times at 0°C before centrifugation at 2000 × *g* and 4°C for 5 min. Radioactivity in 0.1 ml aliquots of supernatant was then determined.

(c) Time course of griseofulvin binding to microtubule protein. PEMG buffer, 500 μl, containing 2 mg microtubule protein/ml and 100 μM griseofulvin in 2% DMF were incubated for the indicated times at 0°C before addition of 100 μl charcoal suspension (24 mg/ml). After incubation at 0°C for 10 min, samples were centrifuged at 2000 × *g* and 4°C for 5 min and radioactivity in 0.1 ml aliquots of supernatant was determined. Controls without protein and charcoal and without protein were treated in the same way.

3375 Tri Carb Liquid Scintillation Counter. Two controls were included for each griseofulvin concentration: 'G' samples with 500 μl PEMG buffer + 10 μl griseofulvin in DMF and 100 μl water in place of the charcoal suspension and 'GC' samples with 500 μl PEMG buffer + 10 μl griseofulvin in DMF. Bound griseofulvin (μM) was calculated as:

$$\frac{\text{T (cpm)} - \text{GC (cpm)}}{\text{G (cpm)}} \times \text{Griseofulvin } (\mu\text{M})$$

SDS–gel electrophoresis was according to the method of Laemmli [9] with 7.5% and 5% acrylamide. Protein was determined by the method of Lowry et al. [10].

3. Results and discussion

3.1. *Griseofulvin binding to unfractionated microtubule protein*

Figure 3 shows the linear relationship between binding ratio and free griseofulvin which was observed under equilibrium conditions over a concentration range 5–35 μM. In contrast, fig.4 shows binding data obtained using the charcoal assay over a griseofulvin concentration-range 5–150 μM. Binding ratios determined by this latter procedure were some 50-fold lower than those determined by equilibrium methods and showed a sharp increase at griseofulvin concentrations, from 40–100 μM. At these concentrations, griseofulvin inhibition of microtubule assembly increases most markedly [1].

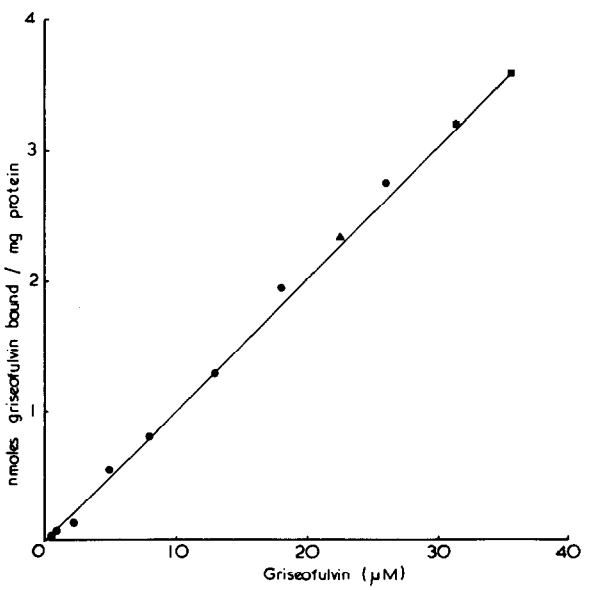

Fig.3. Griseofulvin binding to unfractionated microtubule protein determined under equilibrium conditions. Equilibrium dialysis (●), equilibrium gel-filtration (■), rapid ultrafiltration through an Amicon 'Centriflo' membrane cone (▲).

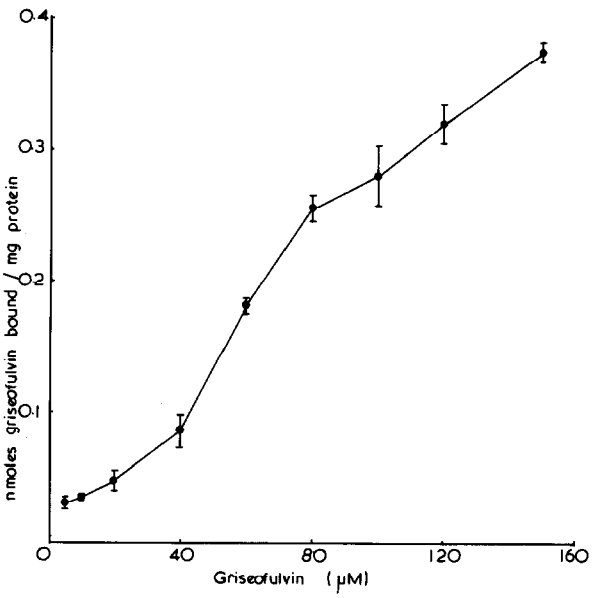

Fig.4. Griseofulvin binding to unfractionated microtubule protein determined by the charcoal procedure described in the text. Each point is the mean of four observations ± one SD.

3.2. *Griseofulvin binding to MAPs and tubulin dimer*

Griseofulvin binding to microtubule protein components separated by DEAE–Sephadex chromatography was measured by the charcoal assay procedure. Figure 5 shows that griseofulvin was bound to a much greater extent by MAPs than by tubulin dimer and that at concentrations of griseofulvin which inhibit microtubule assembly, the binding of griseofulvin by MAPs, but not by tubulin dimer, increased sharply.

Our data indicate that there are at least 2 types of griseofulvin binding to microtubule protein. There is weak non-specific binding which does not appear to affect microtubule assembly since there is very little

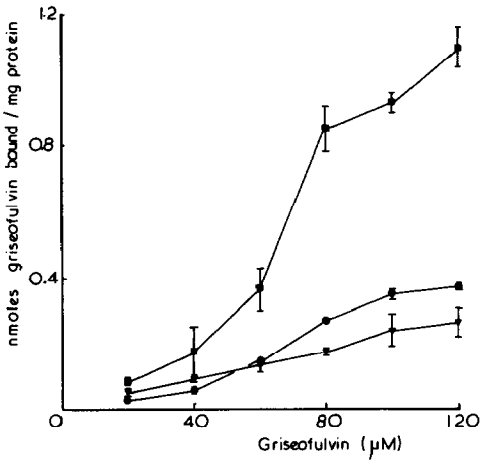

Fig.5. Griseofulvin binding to MAPs and tubulin dimer determined by the charcoal procedure described in the text. MAPs (0.66 mg protein/ml) (■), tubulin dimer (1 mg protein/ml) (▼), unfractionated microtubule protein stored at 4°C for 30 h and exposed to 0.6 M KCl at 4°C for 3 h before desalting on a 1 × 40 cm G-25 Sephadex column (2 mg protein/ml) (●). Each point in the mean of four observations ± one SD.

inhibition of assembly by 30 μM griseofulvin [1] which gives a binding ratio of ~3 nmol/mg microtubule protein under equilibrium conditions. Griseofulvin has a very low stability in water [11] and probably binds readily to hydrophobic regions of proteins.

Sloboda et al. [12] have detected griseofulvin binding to both MAPs and tubulin dimer by Sephadex G-25 chromatography but this is most likely a measure of non-specific binding. In the presence of charcoal much of this non-specifically bound griseofulvin is removed and a specific binding site can be detected in MAPs but not tubulin dimer. The close agreement, between griseofulvin concentrations required to inhibit microtubule assembly and those over which there is a sharp increase in griseofulvin binding to MAPs, strongly suggest that it is griseofulvin binding to MAPs which inhibits microtubule assembly.

To our knowledge, griseofulvin is the first microtubule inhibitor which has been shown to bind to MAPs rather than tubulin dimer. Colchicine [13], vinblastine [14], podophyllotoxin [15], maytansine [16] and oncodazole [17] have all been shown to bind to tubulin dimer. This is consistent with our earlier observation [1] that inhibition of microtubule assembly by griseofulvin is distinctly different from inhibition by these other drugs being characterized by an increase in the lag phase which precedes polymerization.

Acknowledgements

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