A Correlation between *in Vivo* and *in Vitro* Effects of the Microtubule Inhibitors Colchicine, Parbendazole and Nocodazole on Myxamoebae of *Physarum polycephalum*

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The effects of the microtubule inhibitors colchicine, parbendazole and nocodazole on the growth of myxamoebae of *Physarum polycephalum* were closely paralleled by the effects of these drugs on the assembly *in vitro* of purified amoebal microtubule protein. Colchicine at 100 μ M did not inhibit amoebal growth and did not inhibit formation, or depolymerization, of amoebal microtubules. The benzimidazole carbamate derivatives nocodazole and parbendazole were very effective in both inhibiting growth and inhibiting the assembly *in vitro* of amoebal microtubule protein. Parbendazole was the most effective.

INTRODUCTION

Specific microtubule inhibitors are valuable tools for defining the various microtubule-dependent processes in the eukaryotic cell. Such drugs may also be used to induce mitotic arrest which facilitates chromosome studies and is of value in obtaining synchronous cultures. Colchicine, the best known of the anti-microtubule drugs, has, however, been found to be rather ineffective against a number of lower eukaryotes *in vivo* (Jockusch *et al.*, 1971; Haber *et al.*, 1972; Heath, 1975). In studies in which colchicine has been used to induce mitotic arrest in lower eukaryotes (e.g. Cappuccinelli & Ashworth, 1976; Zada-Hames, 1977; Williams, 1980) concentrations in the range 10 to 25 mM have been necessary – concentrations at which colchicine might be expected to affect other non-microtubule-dependent cellular processes (Kuzmich & Zimmerman, 1972). Recently, there have been reports that some of the benzimidazole carbamate group of anti-tubulin agents are much more effective than colchicine against lower eukaryotes *in vivo* and induce mitotic arrest at micromolar concentrations (Mir & Wright, 1978; Cappuccinelli *et al.*, 1979; Williams, 1980; Welker & Williams, 1980). Their mode of action is therefore likely to be highly specific and restricted to the microtubule system. Until now, however, it has not been possible to show directly that the differential drug sensitivity of lower eukaryotes *in vivo* is reflected by the drug sensitivity of lower eukaryotic microtubule protein assembly *in vitro* because it has proved very difficult to purify microtubule protein from such sources.

We have recently been able to achieve an assembly *in vitro* of microtubules in cell extracts of myxamoebae of the slime mould *Physarum polycephalum* and have consequently been able for the first time to purify assembly-competent microtubule protein from a lower eukaryote (Roobol *et al.*, 1980). In this paper we compare the effects of several microtubule inhibitors on the assembly *in vitro* of amoebal and brain microtubule protein. Brain microtubule protein has been used for comparison because nearly all the literature on the interaction of drugs with microtubule protein is based on work with material from this source. We show that the sensitivity *in vitro* of amoebal microtubule protein assembly closely parallels the effectiveness of these drugs in inhibiting the growth of *Physarum polycephalum* amoebae *in*

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in vivo. Of the drugs investigated, the benzimidazole carbamate parbendazole is the most effective both in inhibiting amoebal growth and in inhibiting microtubule protein assembly *in vitro*.

METHODS

Amoebal culture. Myxamoebae of *Physarum polycephalum*, strain CLd, were a gift from Dr J. Dee, University of Leicester, and were grown at 25 °C in a liquid semi-defined medium according to McCullough & Dee (1976). In growth studies, water-insoluble drugs were dissolved in dimethyl sulphoxide (DMSO) and diluted into semi-defined medium to the required final concentration and a final DMSO concentration of 1% (v/v). This low concentration of carrier solvent did not affect amoebal growth. Cell number was determined by direct microscopic observation using a Neubauer counting chamber.

Purification of microtubule protein. Amoebal microtubule protein was purified from cell extracts of mid-exponential phase cultures by two complete cycles of assembly at 30 °C and disassembly at 0 °C (Roobol *et al.*, 1980). Brain microtubule protein was prepared from fresh sheep brain as described previously (Roobol *et al.*, 1976). The pelleted microtubule protein from the second assembly cycle was stored in liquid nitrogen for up to 1 month. Immediately before use, microtubule protein pellets were resuspended in cold assembly buffer [0.1 M-piperazine-*N,N'*-bis-2-ethanesulphonic acid (PIPES), 2 mM-EGTA, 1 mM-MgSO₄, 0.1 mM-EDTA, 1 mM-GTP, 50 µg leupeptin ml⁻¹, pH 6.9] and depolymerized at 0 °C for 30 min followed by centrifugation at 130 000 g for 30 min at 4 °C. The supernatant was retained on ice and used within the following 3 to 5 h during which time there was no loss of polymerization capacity.

Microtubule assembly assays. Microtubule formation was initiated by warming to 30 °C solutions of microtubule proteins in assembly buffer at 0 °C and monitoring the turbidity increase at 400 nm in a Gilford model 250 spectrophotometer (Gaskin *et al.*, 1974). Water-insoluble drugs were first dissolved in DMSO or dimethylformamide (DMF) then diluted into assembly buffer containing the microtubule protein at 0 °C to the required final concentration and a final organic solvent concentration of 2% (v/v). This concentration of DMSO or DMF did not inhibit microtubule assembly *in vitro*. The presence or absence of microtubules during turbidimetric assays was checked by electron microscopy: 5 to 10 µl of sample was placed on a carbon-coated formvar grid, left for 30 s, then displaced and washed with 5 drops of aqueous saturated uranyl acetate and examined in an AEI 801A electron microscope. Protein concentrations were measured by the Lowry method using bovine serum albumin standard solutions.

Materials. Chemicals were Analaar or the purest grade available. Biochemicals and PIPES buffer were purchased from Sigma. Leupeptin was obtained from the Peptide Institute, Osaka, Japan. Methyl benzimidazol-2-yl carbamate (MBC) was a gift from Dr A. Kappas, Greek Nuclear Research Centre, Athens, Greece. Nocodazole was from the Aldrich Chemical Co., Milwaukee, U.S.A. Parbendazole was from Pfizer, Sandwich, Kent.

RESULTS

Microtubule protein prepared from myxamoebae was capable of assembly *in vitro* on warming to 30 °C. Microtubules of normal ultrastructural appearance were formed during this polymerization (Fig. 1).

Effect of colchicine on amoebal growth and assembly in vitro of amoebal microtubule protein

Colchicine at 100 µM did not inhibit growth of myxamoebae (Fig. 2). The same concentration of colchicine was completely ineffective in preventing *in vitro* assembly of amoebal microtubule protein, and the addition of 100 µM-colchicine to amoebal microtubules did not induce depolymerization (Fig. 3). The increase in absorbance immediately after addition of colchicine was due to the absorbance of colchicine alone at the wavelength employed for turbidimetry. The concentration of colchicine used was 10-fold greater than that required to inhibit brain microtubule assembly completely under the same conditions (data not shown).

Effect of benzimidazole derivatives on amoebal growth and assembly in vitro of amoebal microtubule protein

Myxamoebal growth was extremely sensitive to inhibition by some benzimidazole carbamates. Figure 4 shows the effects on growth of nocodazole and parbendazole. Parbendazole was the most effective inhibitor of growth tested and was inhibitory at

Fig. 1. Electron micrograph of a thin section of a pellet of microtubules purified by assembly *in vitro* from *Physarum polycephalum*. Bar marker represents 250 nm.

Fig. 2

Fig. 3

Fig. 2. Effect of colchicine on growth of myxamoebae of *Physarum polycephalum*: O, control; ●, 100 μM-colchicine present in growth medium.

Fig. 3. Effect of colchicine on assembly *in vitro* of amoebal microtubule protein. A stock solution of colchicine was diluted to a final concentration of 100 μM into a solution of amoebal microtubule protein (2.4 mg protein ml⁻¹) in assembly buffer at 0 °C. Solutions were then warmed to 30 °C and turbidity was monitored at 400 nm. (1) Control assembly, to which 100 μM-colchicine was added at the time indicated by the arrow and incubation at 30 °C was continued; (2) 100 μM-colchicine present at the start of the incubation.

Fig. 4. Effect of nocodazole (a) and parbendazole (b) on amoebal growth: ○, control; △, 0.4 μM drug; ●, 2.0 μM drug.

Fig. 5. Effect of nocodazole and parbendazole on assembly *in vitro* of amoebal microtubule protein. Stock solutions of drugs in DMF or DMSO were diluted to the required final concentration and a final concentration of organic solvent of 2% (v/v) into solutions of amoebal microtubule protein (1.45 mg protein ml^{-1}) in assembly buffer at 0 °C. Solutions were then warmed to 30 °C and turbidity was monitored at 400 nm. (1) 2% DMF control; (2) 0.4 μM -nocodazole; (3) 0.4 μM -parbendazole; (4) 2.0 μM -nocodazole; (5) 2.0 μM -parbendazole.

sub-micromolar concentrations. At the same concentrations, these compounds were very effective inhibitors of amoebal microtubule assembly *in vitro* (Fig. 5), with parbendazole again the most effective inhibitor tested. Nocodazole (Hoebeker *et al.*, 1976) and parbendazole (Ireland *et al.*, 1979) are potent inhibitors of brain microtubule assembly, but under the conditions used in our experiments, these drugs were rather less inhibitory for brain microtubule assembly than for amoebal microtubule assembly. The most widely used benzimidazole carbamate, methyl benzimidazol-2-yl carbamate (MBC; Davidse, 1975; Davidse & Flach, 1977; Sheir-Neiss *et al.*, 1978), had little effect on brain microtubule assembly at 50 μM (the highest concentration we could use in solvent conditions which did not inhibit microtubule assembly). However, under the same conditions, this concentration of

MBC caused almost complete inhibition of amoebal microtubule assembly. MBC also inhibited amoebal growth at micromolar concentrations but was rather less effective than parbendazole and nocodazole in this respect.

*Effect of griseofulvin on amoebal growth and assembly in vitro of
amoebal microtubule protein*

Unlike the plasmodial stage of the life cycle of *Physarum* (Gull & Trinci, 1974), the amoebal stage seems fairly insensitive to griseofulvin (Mir & Wright, 1978). Our studies of microtubule assembly *in vitro* have shown that high concentrations ($>30\text{ }\mu\text{M}$) of griseofulvin are required to inhibit assembly and that amoebal microtubule protein is only slightly more sensitive to inhibition by griseofulvin than is brain microtubule protein.

DISCUSSION

We have shown that, with the microtubule inhibitors tested, there is very good correlation between sensitivity *in vivo* and effects on assembly *in vitro* of microtubule protein purified from the same major organism. Also, we have been able to produce unequivocal evidence that microtubules from a lower eukaryote possess a drug sensitivity rather different from that of higher eukaryote microtubules. It is, therefore, no longer necessary to evoke differences in uptake or subsequent metabolic inactivation to explain this differential sensitivity towards microtubule inhibitors. Of particular interest is the lack of sensitivity of amoebae and amoebal microtubule protein towards colchicine. Preliminary colchicine binding assays (Roobol *et al.*, 1980) have shown that the binding ratio for amoebal microtubule protein at $100\text{ }\mu\text{M}$ -colchicine is some 50-fold lower than the binding ratio for brain microtubule protein determined under the same conditions. Since extremely high concentrations of colchicine are required to disrupt microtubule-dependent processes in lower eukaryotes, we suggest that this is not the drug of choice for use in studies of microtubule-dependent processes in these organisms and that at these high concentrations the effects of the drug may not be restricted to the microtubule system.

The benzimidazole carbamates nocodazole and parbendazole are the most effective inhibitors we have studied. The close correlation of concentrations effective for inhibition of growth and of microtubule formation *in vitro*, together with the low concentrations required, strongly suggest that the action of these drugs *in vivo* is restricted to the microtubule system of the organism. These drugs are therefore the drugs of choice for disrupting microtubule-dependent processes in *Physarum* and other lower eukaryotes. Finally, in the context of using microtubule inhibitors to achieve synchronized cell cultures, we have noted a quite large discrepancy between measurements of parbendazole binding to tubulin by an equilibrium method and measurement by a non-equilibrium method. These data indicate that parbendazole dissociates rapidly from tubulin once unbound drug is removed (see also Hoebeke *et al.*, 1976). This contrasts with observations with colchicine which dissociates very slowly ($t_{1/2} = 25\text{ h}$ at $37\text{ }^{\circ}\text{C}$; Wilson, 1975) from the tubulin-colchicine complex. We conclude, therefore, that parbendazole would be more suitable for inducing cell synchrony than colchicine since the block in mitosis should be rapidly reversible with the former drug.

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