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INHIBITION OF TUBULIN POLYMERIZATION BY MEBENDAZOLE

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SUMMARY: The interaction of Mebendazole (methyl-5-benzoyl benzimidazole-2-carbamate), a new antihelminthic drug, with tubulin was studied. Ultramicroscopic and turbidimetric evidence shows an inhibitory effect of Mebendazole on the "in vitro" polymerization of tubulin. Scatchard plot analysis shows a single binding site for Mebendazole per tubulin dimer. This site has an affinity constant of 2.8×10^5 M^{-1}. Competition experiments demonstrate that this binding site is the same as for Colchicine, even when both compounds are not chemically related. Mebendazole is proposed as a useful tool for the study of tubulin assembly.

INTRODUCTION

Microtubules are conspicuous organelles, found in all eukariotic cells, formed primarily by tubulin as the repetitive subunit. They have been implicated in a variety of cell phenomena such as chromosome movement (1), vesicular transport (2), ciliary and flagellary motion (3) and cellular shape maintenance (4,5) among others. It is generally accepted that microtubules function through control mechanisms on their polymerization state. Several compounds such as Colchicine (6-8), Vinca alkaloids and Podophyllotoxin (8,9), inhibit this polymerization by direct interaction with tubulin.

Recently, Hoebeke et al (10) reported the inhibitory properties of Oncodazole, an antitumoral drug, on the "in vitro" polymerization of tubulin. A similar compound, Mebendazole (Fig. 1) used as a potent and broad spectrum antihelminthic, has been tested on some larval and adult forms of nematodes and cestodes (11-14), producing two primary effects that suggest an action on the microtubular system of those worms: 1) Blockage of vesicle movement in secretory

ABREVIATIONS USED: MES: 2(n-morpholino) ethane sulfonic acid; EGTA: Ethylenglycol-bis-(B-amino-ethyl ether) N,N-tetraaetic acid; DMSO: Dimethyl sulfoxide.

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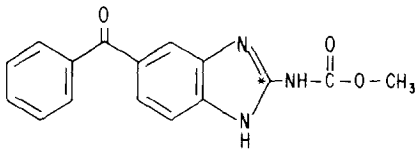

Fig. 1: The chemical structure of Mebendazole. The asterisk indicates the position of the radioactive carbon atom.

cells; 2) Ultrastructural loss of citoplasmic microtubules. We report here, data on the direct interaction of Mebendazole with tubulin, as an approach to understand its mechanism of action.

MATERIALS AND METHODS

Drugs and chemicals

GTP was purchased from Sigma; [^{3}H] Colchicine from New England Nuclear; Cold and radioactive [^{14}C] Mebendazole were kindly provided by Dr. H. Van Den Bosch, from Janssen Pharmaceutica. Sephadex G-100 was obtained from Pharmacia Fine Chemicals. All other chemicals were of analytical grade.

Tubulin preparation

Tubulin was extracted from bovine brain in two polymerization-depolymerization cycles, following the method reported by Shelanski et al (15). Protein cycled once was stored at -20°C in a buffer solution containing 0.1 M MES, 0.5 mM $MgCl_2$, 0.1 mM EGTA pH 6.5 and 25% glycerol. Before use, the protein was cycled once more and resuspended in the same buffer without glycerol for the polymerization experiments or in 1 mM phosphate pH 6.8 for all binding experiments. Protein concentration was determined by using the method of Lowry et al (16), and adjusted to 1-2 mg/ml. Protein was always used within one week after extraction. Tubulin was more than 80% of the total protein in a twice cycled sample, as determined by scanning of polyacrylamide electrophoresis gels.

Polymerization experiments

Mebendazole was added in these, as in all experiments, as 5 µl of a DMSO solution. One ml aliquots of tubulin were preincubated for 30 minutes at 37°C either with Mebendazole or DMSO alone for the control samples. Microtubule formation was followed measuring the GTP-induced increase in turbidity of the samples at 350 nm on a PMQ_{II}Zeiss spectrophotometer. During measurements, the samples were mantained at 37°C on a thermostated cuvette holder. Each sample was analized by electron microscopy at the end of the experiment.

Negative staining for electron microscopy

Adsorption of tubulin samples were done on double layered (formvar-carbon) electron microscope grids. Samples were mantained in contact with the grids for 15-30 seconds and stained for 15 seconds, using a 1% (w/v) Uranyl Acetate solution.

Binding experiments

a) Column chromatography: 1 ml of tubulin was incubated 30-60 minutes at 37°C in the presence of a given concentration of [^{14}C] Mebendazole.

After cooling to 4°C, the sample was applied to a column of Sephadex G-100 mantained at the same temperature. One ml fractions were collected, and protein was determined in each fraction. Radioactivity was measured evaporating 100 µl aliquots from each tube on glass-fiber discs, suspended in a toluene-fluor mixture and radioactivity counted in a Packard Tri-carb liquid scintillator spectrometer.

b) Equilibrium dialysis: These were carried out in a two chamber system, consisting of an inner compartment of dialysis tubing containing 1 ml of tubulin and [^{14}C] Mebendazole at a given concentration. The tubing was immersed in a chamber containing 10 ml of 1 mM phosphate buffer including [^{14}C] Mebendazole at the same concentration as inside. Aliquots of 50 µl were taken from the dialysis tubing at 0 and 120 minutes and from the external chamber at 0, 30, 60, 90 and 120 minutes and radioactivity was determined to asses equilibrium conditions. The external chamber was mantained at 37°C and provided with constant stirring. Competition experiments were carried out in a similar way but adding colchicine at equal concentrations to both compartments.

RESULTS AND DISCUSSION

The twice cycled preparation of tubulin, showed very reproducible polymerization kinetics: The maximal turbidity value for the control samples, varied less than 5%. Dimethyl sulfoxide, the vehicle used to solubilize Mebendazole, had not a noticeable effect on the assembly of tubulin, at the concentrations used.

Mebendazole had an inhibitory effect on the polymerization of tubulin (Fig. 2). A concentration of 9 µM was sufficient to produce 50% of

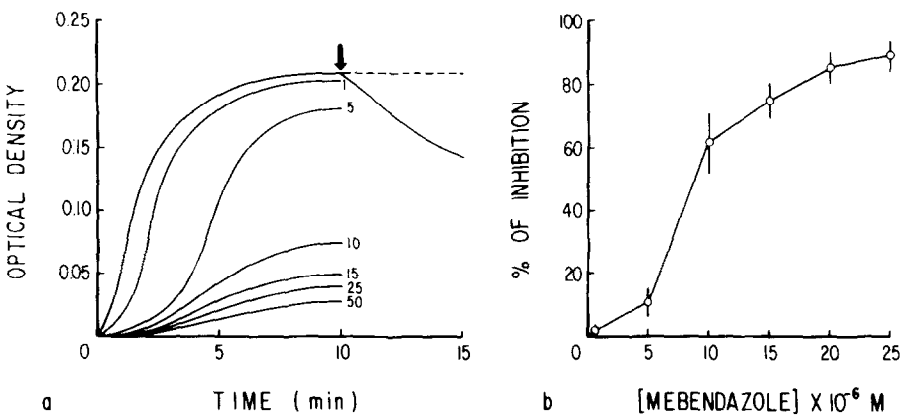

Fig. 2: The effect of Mebendazole on the "in vitro" polymerization of tubulin. After preincubation (see Materials and Methods), tubulin samples were induced to polymerize by addition of 1 mM GTP.
a) The numbers at the end of lines show the concentration of Mebendazole (µM). The arrow indicates a subsequent addition of 50 µM Mebendazole.
b) 100% was defined as the average of the maximal turbidity obtained in control samples. The percent of inhibition is also referred to the maximal turbidity value of treated samples.

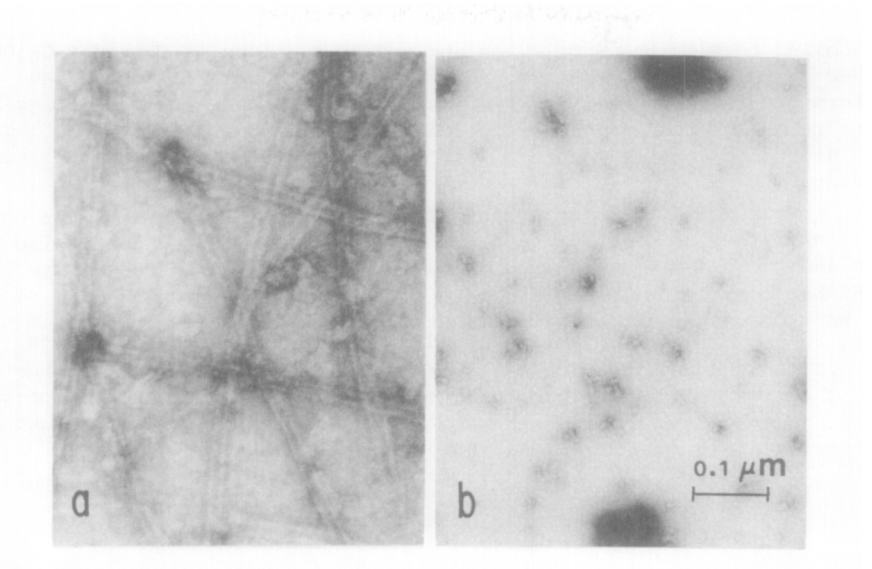

Fig. 3: Electron microscopic images of a control a) and a treated sample b). The treated sample had 50 μM Mebendazole.

inhibition (Fig. 2b). The maximal inhibition produced by Mebendazole was of about 90% when compared with the turbidity obtained in control samples. Colchicine produced also a maximal inhibition nearly to 90%. The electron microscopic images observed in samples from each assay correlated quite well with the turbidimetric results (Fig. 3). The inhibition seems to have two features: 1) An enlargement in the lag period of the polymerization; 2) A decrease in the maximal polymerization with respect to the control.

A slow decrease in turbidity was obtained when Mebendazole was added on preformed microtubules (Fig. 2a), indicating that Mebendazole not only inhibited but also reversed microtubule assembly "in vitro". This is in agreement with results reported by Borisy *et al* (18), showing that 100 μM of colchicine depolymerized preformed microtubules.

The above results suggest that Mebendazole interacts directly with tubulin, therefore, a first measurement of its binding to the protein was assayed. Figure 4, shows the elution profile of a tubulin preparation incubated in the presence of radioactive Mebendazole. A peak of radioactivity overlapped the protein peak representing the bound Mebendazole. The second peak

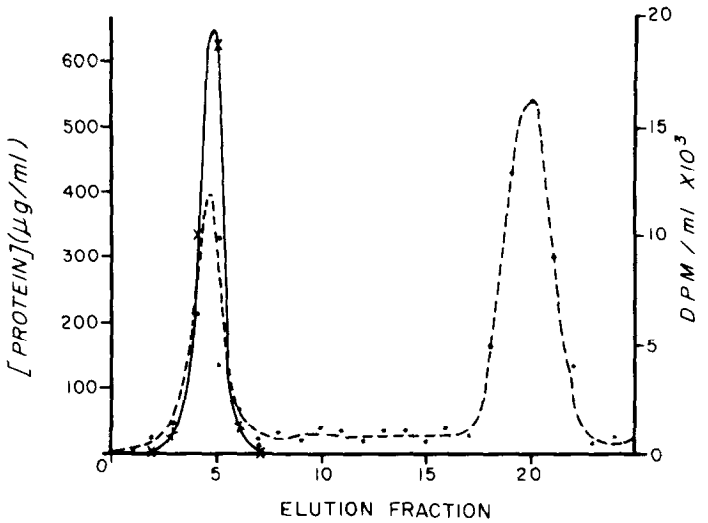

Fig. 4: Elution profile of a preparation of tubulin preincubated in the presence of Mebendazole during 30-60 min. (————) protein; (------) radioactivity.

represents the unbound Mebendazole. No differences were observed varying the preincubation times between 30 to 60 minutes, suggesting a rapid equilibrium of binding.

The number of binding sites per tubulin dimer was subsequently estimated by equilibrium dialysis determinations. As shown in figure 5a, binding

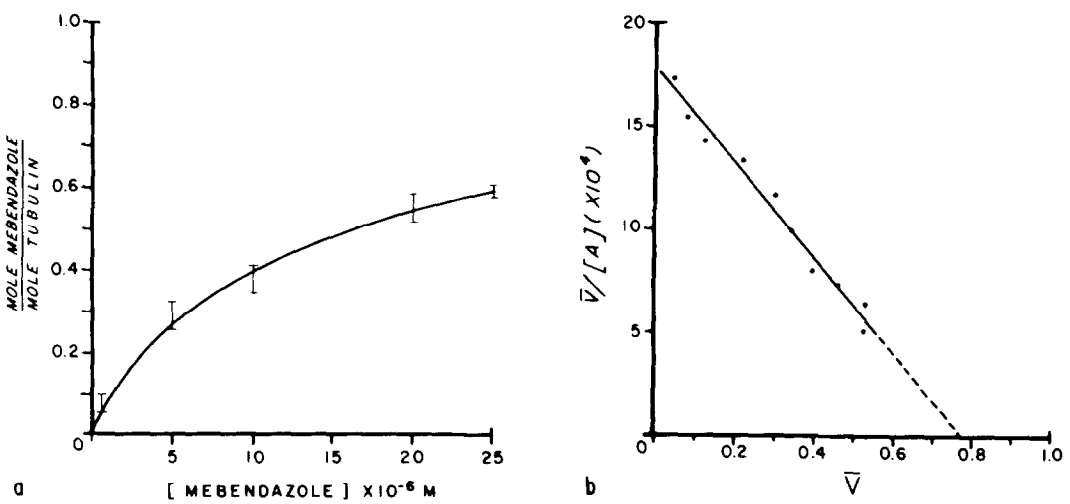

Fig. 5: Saturation curve (a) and Scatchard plot (b), of a preparation of tubulin incubated with variable concentrations of Mebendazole (r^2 = 0.95).

of Mebendazole to tubulin within the range of concentrations tested, seems to be a simple saturation process. Plotting the same data according to Scatchard (17), the number of binding sites per tubulin dimer was 0.78. This value is similar to those reported for colchicine, varying from 0.6 to 1.07 (19-21). The affinity constant of binding was 2.8×10^5 M^{-1}.

Competition experiments between Mebendazole and Colchicine indicated that both drugs share the same binding site on tubulin. This is in agreement with data reported for a similar compound (10). However, the biphasic profile of inhibition (see figure 2b), could suggest that tubulin posses another class of binding site for Mebendazole not detected here. Additional studied employing different incubation conditions are needed to demonstrate this possibility.

The inhibition of tubulin assembly produced by Mebendazole, can explain the disappearance of labile microtubules and the concomitant impairing of vesicle movement, observed in secretory cells of parasites treated with this drug (11-13). Additionally, the reported differences in susceptibility to Mebendazole between parasite and host cells, can not be atributed to differences in both microtubular systems. The results presented here, seem to support one of the alternative explanations proposed by Borgers et al (12), in the sense that Mebendazole may have a different rate of degradation in both organisms. However, an unequal uptake of Mebendazole can not be excluded.

Mebendazole and its relative Oncodazole, form a new group of simple chemicals potentially useful in the study of tubulin assembly and function.

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