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Role of B-ring of colchicine in its binding to Zn(II)-induced tubulin-sheets

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Abstract. Colchicine-tubulin dimer complex, a potent inhibitor of normal microtubule assembly undergoes extensive self-assembly in the presence of 1×10^{-4} M zinc sulphate. Polymers assembled from colchicine-tubulin dimer complexes are sensitive to cold.

Although colchicine can be accomodated within the polymeric structure, the drug cannot bind to tubulin subunits in the intact polymers. This is evidenced by the fact that (a) the colchicine binding activity of tubulin is lost when allowed to polymerize with zinc sulphate, (b) the loss in colchicine binding could be prevented by preincubation of tubulin with 1×10^{-3} M $CaCl_2$ or 1×10^{-5} M vinblastine sulphate and finally (c) no loss in colchicine binding activity is found when tubulin is kept at a concentration far below the critical concentration for polymerization. Unlike colchicine, its B-ring analogues desacetamido colchicine (devoid of the B-ring subtituent) and 2-methoxy-5- (2′,3′,4′-trimethoxyphenyl) tropone (devoid of the B-ring) can bind to tubulin subunits in the intact polymers.

Thus we conclude that the colchicine binding domain on the tubulin molecule is mostly (if not completely) exposed in the Zn(II)-induced polymers and the B-ring substituent plays a major role in determining the binding ability of a colchicine analogue to tubulin in the intact Zn(II)-induced sheets.

Keywords. Colchicine-tubulin dimer complex; B-ring; tubulin sheets.

Introduction

The alkaloid colchicine was known as an inhibitor of mitosis long before the discovery of microtubules (Dustin, 1978). Extensive studies on the mechanism of action of colchicine have established that it exerts its antimitotic action through binding to tubulin. Although tubulin can bind colchicine, however, tubulin within the assembled microtubule cannot bind colchicine (Wilson and Meza, 1973). *In vitro* microtubule assembly is inhibited by substoichiometric concentrations of colchicine; half maximal inhibition occurs when only 2% of the unpolymerized tubulin is complexed with drug (Olmsted and Borisy, 1973; Wilson and Bryan, 1974; Margolis and Wilson, 1977). A similar mechanism also appears to be applicable to *in vivo* poisoning; in the case of mitosis, colchicine exerts its influence when only a small fraction of the cellular pool of tubulin is complexed with drug.

This substoichiometric poisoning of microtubule assembly has been explained by the Capping of the growing end of microtubules by the drug-tubulin complex (Margolis and Wilson, 1977). Sternlicht and Ringel (1979) explained the above phenomena on the basis of a copolymerization model in which both tubulin and colchicine-tubulin dimer (CD) complex are incorporated into microtubules.

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Abbreviations used: CD, Colchicine-tubulin dimer; GTP, Guanosine-5′–triphosphate; SDS, sodium dodecyl sulphate; pp (CH_2) pG, guanylyl-5′–methylene diphosphonate.

According to their model, incorporated CD complexes decrease the affinity of the microtubule end for further addition of tubulin dimer. It has been reported from this laboratory (Banerjee *et al*., 1982) that even only CD complexes could be polymerized in the presence of zinc sulphate. Analysis of the polymerized products harvested in the presence of [^{3}H]-colchicine indicates the incorporation of 0·5 mol of colchicine per mol of tubulin. From this observation, it is apparent that the colchicine binding site on tubulin might be different from the tubulin-tubulin interaction sites involved in the process of Zn(II)-induced assembly. Thus, the question may arise as to whether the colchicine binding site in the Zn(II)-induced polymer is buried, if not, whether colchicine could bind to intact Zn(II)-induced polymer. Our studies on this aspect revealed that the preformed sheets cannot bind colchicine. However, colchicine analogues with no substituents in the B-ring (of colchicine) (figure 7) such as desacetamido colchicine and 2-methoxy-5-(2′,3′,4′–trimethoxyphenyl) tropone (devoid of B-ring) could bind to intact Zn(II)-induced sheets suggesting that the size of the B-ring of colchicine plays an important role in determining the binding properties of colchicine to Zn(II)-induced sheets.

Materials and methods

Materials

Colchicine, Guanosine-5′-triphosphate (GTP) (Grade IIS), 2-(N-morpholino)ethane-sulphonic acid (MES) and sodium dodecyl sulphate (SDS) were products of Sigma Chemical Company, St. Louis, Missouri, USA. Colcemid was obtained from K and K. B-ring modified analogues of colchicine were the kind gifts of Dr. Thomas J. Fitzgerald of School of Pharmacy, Florida A and M University, Tallahasee, Florida, USA. DEAE-cellulose (DE 52) and DEAE-cellulose paper (DE 81) were obtained from Whatman Ltd., England. [^{3}H]-Colchicine (ring C, methoxy ^{3}H) was a product of New England Nuclear Corporation, USA. Fresh goat brains were obtained from a local slaughter house.

Purification of tubulin

Tubulin was purified from goat brain in PMG buffer (10 mM potassium phosphate, pH 7·0; 10 mM $MgCl_2$; 0·1 mM GTP) according to Weisenberg *et al*. (1968), except that DEAE-cellulose was used instead of DEAE-sephadex. The active fractions as judged from a colchicine-binding assay (Williams and Wolf, 1972) were pooled, concentrated by overnight dialysis at 0°C against 100 volumes of 8 M glycerol in buffer A (100 mM MES, pH 6·4; 0·5 mM $MgCl_2$; 1·0 mM GTP) and stored at – 70°C. The purity of tubulin was checked by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970). Protein concentrations were determined as described by Lowry *et al*. (1951) using bovine serum albumin as standard.

Spectrophotometric assay of tubulin assembly

Assembly reactions were performed at 37°C in buffer A and monitored spectrophotometrically in a Cary 17D spectrophotometer recording at 400 nm. A wavelength of

400 nm was selected so as to minimize the absorption of colchicine. Temperature of the cuvettee chamber was maintained at 37°C using an LKB model 2209 circulator.

[^{3}H]-Colchicine-binding assay

[^{3}H] -Colchicine binding assay was performed according to the DEAE-cellulose filter disc method of Weisenberg *et al*. (1968) as modified by Williams and Wolf (1972).

Fluorometric assay for drug binding

The binding of unlabelled drug (colchicine, desacetamido colchicine and 2-methoxy-5-(2*'*,3*'*,4*'*-trimethoxyphenyl) tropone to tubulin was assayed by the fluorometric method according to Bhattacharyya and Wolff (1974). Fluorescence was measured at 37°C in a Perkin Elmer model MPF 44B Spectrofluorometer. Samples were excited at a wavelength of 380 nm, emission was scanned from 390–550 nm.

Electron microscopy

Electron microscopy was done according to Johnson and Borisy (1979) using 1% uranyl acetate.

Results

Colchicine binding activity of tubulin upon assembly in the presence of Zn(II)

It has been demonstrated from this laboratory that suprastoichiometric concentrations of colchicine do not inhibit the assembly of tubulin in the presence of zinc sulphate (Banerjee *et al*., 1982). We also demonstrated that even CD complexes are capable of assembly in the presence of 1×10^{-4} M zinc sulphate. Electron microscopic examination of the assembly products obtained from pure tubulins by Zn(II) are shown in figure 1. Negatively stained samples showed that at the highest Zn(II) concentration (1×10^{-4}M) the polymers are sheets in nature. This is in agreement with the results of others (Larsson *et al*., 1976; Wallin *et al*., 1977; Gaskin and Kress, 1977; Haskins *et al*., 1980). The colchicine content of the Zn(II)-induced polymers harvested from the [^{3}H]-labelled CD complexes have been determined and found to be 0·4–0·5 mol of colchicine per mol of tubulin dimer. It appears from this that the colchicine-binding site and the tubulin-tubulin interaction sites are different in the case of Zn(II)-induced assembly. The incorporation of the colchicine molecule into the Zn(II)-induced polymer further indicates that the polymer could accommodate the drug molecule within its lattice. Thus, the question may arise as to whether the preformed polymers bind colchicine. Therefore, experiments were designed to study the colchicine-binding property of 6S tubulin after assembly in the presence of zinc sulphate.

For this purpose, aliquots of tubulin were incubated with various concentrations of zinc sulphate at 37°C for 30 min; samples were subsequently incubated further with [^{3}H]-colchicine (1×10^{-6} M) at 37°C for 30 min and the bound colchicine was

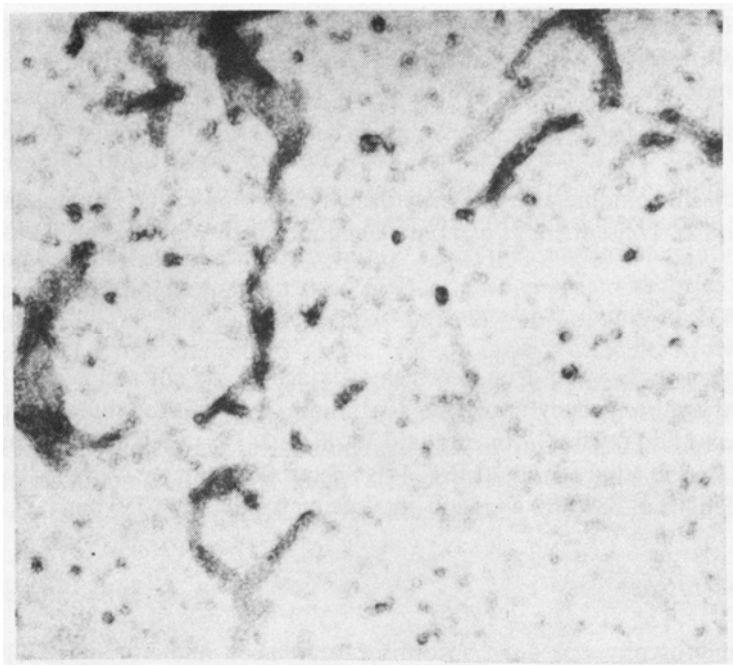

Figure 1. Electron micrographs of Zn(II)-tubulin polymer. DEAE-tubulin (0·7 mg/ml) in buffer A was polymerized at 37°C for 30 min in the presence of 1×10^{-4} M $ZnSO_4$. A 5 μl aliquot of the polymerized sample was loaded onto a 400 mesh copper grid and negatively stained with 1% uranyl acetate as described in the 'materials and methods'. Grids were examined in a Phillips Model *EM301* electron microscope under an accelerating voltage of 60 KV (× 19,000).

assayed (figure 2). As shown in figure 2 preincubation of tubulin with $ZnSO_4$ at concentrations upto 1×10^{-5} M had no effect on the colchicine-binding activity while at concentrations above 2×10^{-5} M it caused a gradual inhibition of colchicine binding activity. The maximum inhibition occurred in the range of 5×10^{-5}–l × 10^{-4} M $ZnSO_4$ and further increase in the zinc sulphate concentration did not result in any further increase in the extent of inhibition. Infact, Zn(II) at 1×10^{-5} M does not induce assembly but the induction of assembly occurs at a Zn(II) concentration above 2×10^{-5} M (figure 3). It is, therefore, conceivable that the inhibition of colchicine binding activity of tubulin upon incubation with $ZnSO_4$ is a consequence of polymerization of tubulin. That this inhibition in colchicine binding is not due to the blocking of sulphydryl groups is evidenced by the fact that the inclusion of mercaptoethanol into the reaction mixture could not overcome the inhibition. That the inhibition is not due to the inactivation (or denaturation) of tubulin is shown by the total recovery of the colchicine-binding activity on treatment with Zn(II)-chelators like ethylenediamine tetraacetic acid or *o*-phenanthroline (data not shown). Moreover, this inhibition in colchicine binding could be prevented by incubation of tubulin with its assembly inhibitor such as 1×10^{-3} M calcium chloride or 1×10^{-5} M vinblastine sulphate (prior to the addition of zinc sulphate). The above results indicate that the loss of colchicine-binding could be either due to the polymerization of tubulin or due to an alteration in the colchicine-binding site by the

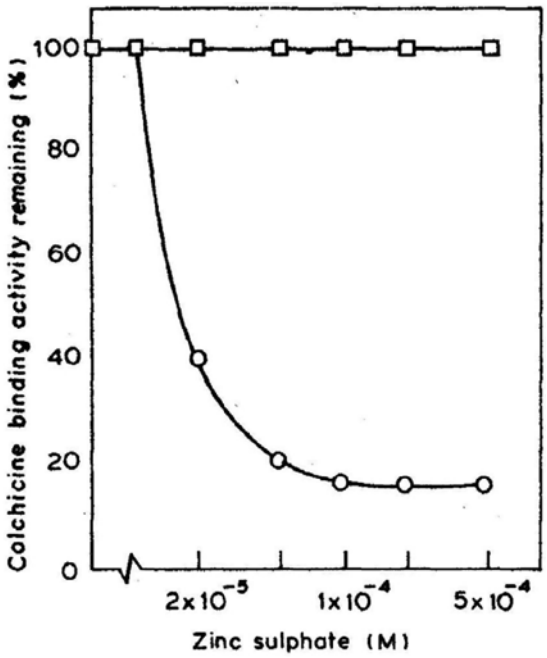

Figure 2. Colchicine binding activity of tubulin upon assembly in the presence of $ZnSO_4$.
Aliquots of DEAE-purified tubulin (0·75 mg/ml) in buffer A were incubated with different concentrations of $ZnSO_4$ at 37°C for 30 min. Colchicine binding activity of the samples were subsequently determined by incubating the samples with [^{3}H]-colchicine (1×10^{-6}M) at 37°C for 30 min. Colchicine binding assays were carried out with DE-81 filter paper as described under 'materials and methods'. Binding activity of each sample was expressed as a per cent activity remaining compared to a sample containing no $ZnSO_4$ (O). A second set of samples was preincubated with either 1×10^{-3} M $CaCl_2$ (❑) or 1×10^{-5} M vinblastine sulphate (❑) prior to the incubation with $ZnSO_4$. For non polymerizing condition; aliquots of DEAE-tubulin (0·05 mg/ml) in buffer A were incubated with different concentration of $ZnSO_4$ at 37°C for 30 min and the colchicine binding activity of the samples were determined (❑) (taken from Banerjee, 1981).

binding of Zn(II) to tubulin. However, the latter possibility seems to be unlikely since Zn(II) exhibits no inhibitory effect when tubulin concentration was kept far below the critical concentration* for polymerization (figure 2). Moreover, the loss in colchicine-binding activity has been found to correlate with the loss of tubulin from the supernatant (table 1). A slight variation in the results obtained by two different methods might result due to the binding of colchicine to ends of tubulin sheets. All these data clearly indicate that the intact Zn(II)-induced polymer binds colchicine very poorly.

Studies with colchicine analogue

From the previous results, it is clear that although the colchicine molecule could be accomodated with the Zn(II)-indueed polymers, the drug binds to intact sheets very

*Critical concentrations for polymerization of DEAE-purified tubulin in the presence and absence of 1×10^{-4} M $ZnSO_4$ are 0·12 mg/ml and 1·4 mg/ml respectively (Banerjee *et al*., 1982).

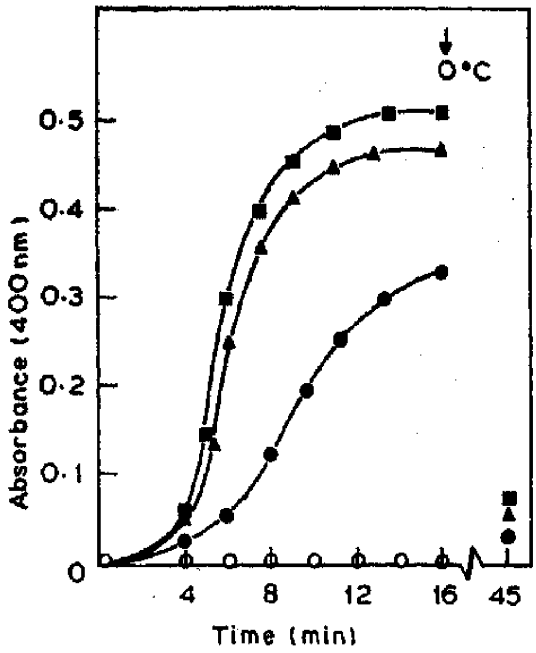

Figure 3. Assembly of DEAE-tubulin at different Zn (II) concentrations.
DEAE-tubulin (0·7 mg/ml) in buffer A was incubated at 37°C in the presence of different concentrations of $ZnSO_4$ and the polymerization was monitored turbidimetrically at 400 nm. $ZnSO_4$ concentrations were as follows: None or 1×10^{-5} M (O), 2×10^{-5} M (●), 5×10^{-5} M (▲) and 1×10^{-4} M (■). Arrow indicates the time when cuvette was placed at 0°C.

Table 1. Correlation of the colchicine binding inhibition with the amount of protein pelleted by centrifugation.

Tubulin concentration (mg/ml)	Colchicine binding[a]		Inhibition in colchicine binding (%)	Protein concentration (mg/ml)		
	Unpolymerized	Polymerized		Before polymeri-zation	After pelleting down the polymer[b]	Protein pelleted (%)
1·2	5140	640	87·6	1·2	0·10	92
0·8	3749	580	84·6	0·8	0·11	87
0·4	2032	632	69	0·4	0·10	75

[a]Counts/min/0·l ml reaction mixture. [b]Supernatants were examined for protein.
Aliquots of DEAE-purified brain tubulin were incubated at different protein concentrations in bluffer A with or without 1×10^{-4} M $ZnSO_4$ at 37°C for 30 min. In one set of experiments, colchicine binding of samples were determined by further incubating the samples with [^{3}H]-colchicine (1×10^{-6} M) at 37°C for 30 min. In the other set of experiments, samples polymerized in the presence of $ZnSO_4$ were centrifuged at 120,000 *g* for 30 min at 25°C to pellet down the polymer.

poorly. Possible explanation for the burial of the colchicine-binding site in the Zn(II)-induced polymer might be that although the tubulin-tubulin interaction sites and the colchicine-binding sites in native tubulin are different, it is possible that the colchicine-binding site and the tubulin-tubulin interaction sites involved in sheet

formation may overlap spatially. An alternate mechanism might be that colchicine binding to tubulin which requires a change in conformation of the tubulin molecule (Ventilla *et al*., 1972; Garland, 1978; Lambeir and Engelborghs, 1981; Detrich *et al*., 1982; Andreu and Timasheff, 1982a) is hindered due to a restricted movement of tubulin in the assembled state compared to the free state.

To test these possibilities, we chose certain colchicine analogues *viz*., desacetamido colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone which are known to bind tubulin at the same site where colchicine binds (Ray *et al*., 1981). Further, they are structurally related to, yet less bulky than colchicine (figure 7).

Effect of colchicine analogues on the Zn(II) tubulin sheet formation: We have demonstrated that colchicine is ineffective at inhibiting the Zn(II)-tubulin sheet formation (Banerjee *et al*., 1982). To test the effect of colchicine analogues on Zn(II)-tubulin sheet formation, we preincubated brain tubulin with 1×10^{-5} M of either analogue at 37°C for 30 min. Assembly was subsequently initiated by the addition of 1×10^{-4} M $ZnSO_4$ (figure 4). As shown in the figure, tubulin complexed with desacetamido colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone could polymerize well in the presence of $ZnSO_4$. In the case of the drug-treated samples the rate of assembly was less although the steady state turbidities were almost the same as that of the control sample. In this context it should be mentioned that similar behaviours of colchicine and podophyllotoxin in the assembly of microtubule protein in the presence of a GTP analogue, guanylyl-5′-methylene diphosphonate (pp (CH_2)

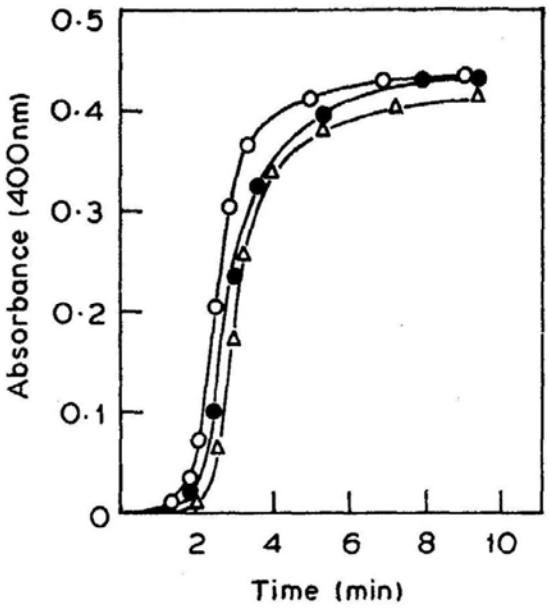

Figure 4. Effect of colchicine analogues on the Zn (II)-tubulin assembly.

Aliquots of DEAE-purified brain tubulin (0·7 mg/ml) in buffer A were incubated at 37°C for 30 min either alone (O) or in the presence of 1×10^{-4} M desacetamido colchicine (Δ) or 1×10^{-4} M 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone (●). Samples were subsequently polymerized at 37°C in the presence of 1×10^{-4} M $ZnSO_4$.

pG) were first reported by Sandoval and Weber (1979). Recently it has been reported that the tubulin-colchicine complex undergoes polymerization under the same *in vitro* conditions which promote microtubule assembly, however, the resulting polymers showed amorphous structure as well as large aggregates which appear to be fibrous and did not reveal any organized structure (Saltarelli and Pantaloni, 1982; Andreu and Timasheff, 1982a,b).

Effect of colchicine analogues on the preformed Zn(II)-tubulin sheets: In order to study the effect of colchicine analogues on the preformed Zn(II)-tubulin sheets, tubulin was assembled in the presence of 1×10^{-4} M $ZnSO_4$ at 37°C for 30 min. Steady state polymers were further incubated with 1×10^{-4} M drug at 37°C and the turbidity was monitored. It has been observed that the turbidity remained almost unchanged for about an hour in the case of desacetamido colchicine and 2-methoxy-5-(2′,3′,4′–trimethoxyphenyl) tropone treated samples whereas there are about 15–20% decrease in the optical density in the case of colchicine treated one. These results indicate that Zn(II)-tubulin sheets are stable to treatment with desacetamido colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone whereas they are partially susceptible toward colchicine (data not shown).

Binding of colchicine analogues to Zn(II)-tubulin sheets: Unlike colchicine, its two B-ring analogues, desacetamido colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone bind to tubulin at a faster rate (Ray *et al*., 1981). Like colchicine, the analogues upon binding to tubulin exhibit fluorescence emission maxima at 430 nm. To study the binding of these analogues to intact Zn(II)-induced polymers tubulin was assembled to steady state in the presence of zinc sulphate (1×10^{-4} M) and subsequently incubated further with 1×10^{-4} M drug at 37°C for 15 min. The fluorescence spectra of the drug treated polymerized samples were scanned and compared with an identically treated sample of unpolymerized tubulin. The results of such a study employing colchicine, desacetamido colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone have been depicted in figure 5. As evident from the figure, there is a marked difference between the colchicine fluorescence of a sample of unpolymerized tubulin and that of an identical sample polymerized to steady state in the presence of zinc sulphate. The fluorescence of the polymerized sample is about 30–35% of that of the unpolymerized one (figure 5A). In fact, this 30–35% of fluorescence is due to the free tubulin after the colchicine-treatment of polymers. The total free tubulin is the sum of the tubulin present in equilibrium with the polymers at steady state (critical protein concentration for polymerization) plus the amount derived from the depolymerization of polymers by colchicine treatment (1×10^{-4} M). This result is consistent with the conclusion obtained from figure 2 that the Zn(II)-induced polymers cannot bind colchicine. In contrast, the fluorescence emission spectra of both desacetamido colchicine (figure 5B) and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone (figure 5C) were almost the same with either polymerized or unpolymerized sample of tubulin. These results clearly indicate that unlike colchicine, its two B-ring analogues could bind to intact Zn(II)-induced polymers of tubulin. In all cases, the fluorescence emission intensity was corrected for protein in the absence of drug.

The same conclusion is further confirmed by the [3H]-colchicine binding assay. It

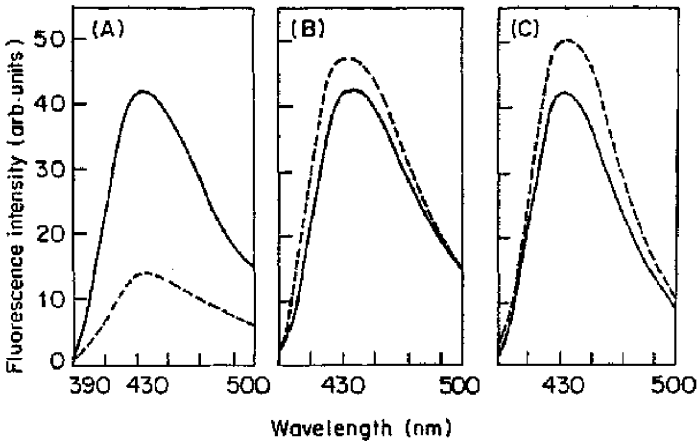

Figure 5. Binding of colchicine and its analogues to tubulin before and after assembly in the presence of $ZnSO_4$. Aliquots of DEAE-purified tubulin (0·98 mg/ml) in buffer A were polymerized in the presence of 1×10^{-4} M $ZnSO_4$ at 37°C for 30 min. These polymerized samples together with polymerized tubulin samples were incubated with 1×10^{-4} M drug at 37°C for 15 min. Fluorescence emission spectra of the samples were recorded subsequently from 390–500 nm after exciting at 350 nm. Fluorescence of both polymerized (---) and unpolymerized (—) samples are presented (A), Colchicine; (B), desacetamido colchicine; (C), 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone.

Appropriate corrections were made for scattering of assembled tubulin in the absence of drug (Banerjee, 1981).

has been reported from this laboratory (Ray *et al*., 1981) that colcemid, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone bind to tubulin at the same site at which colchicine binds. Thus it has been possible to study the binding of these B-ring analogues to the intact Zn(II)-induced polymers in an experiment in which [^{3}H]-colchicine could be used as a probe to detect the presence of drug molecules bound at the colchicine binding site on tubulin.

In this study, tubulin was polymerized in the presence of 1×10^{-4} M zinc sulphate to steady state and the polymers were subsequently incubated further with 1×10^{-4} M unlabelled drugs at 37°C for 30 min. The drug treated polymers were pelleted by warm (25°C) centrifugation at 120,000 *g* for 30 min. The pellets were resuspended in buffer A, depolymerized with EDTA and were incubated with [^{3}H]-colchicine. The course of [^{3}H]-colchicine binding was followed in each case and was compared to that of a control pellet obtained without treatment by any drug. The results of such an experiment employing colchicine, colcemid, desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone have been summarized in figure 6. As shown in the figure, the uptake of [^{3}H]-colchicine for colchicine and colcemid-treated polymer pellets are almost identical to that of the untreated control pellet, which clearly indicates that those two drugs cannot bind to the intact polymers significantly. In contrast, the uptake of radioactivity for desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone-treated pellets were markedly inhibited as compared to that of the control pellet. A small increase in the uptake of radioactivity for the 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone-treated sample is probably due to the reversible binding property of this drug to tubulin (Ray *et al*. 1981) so that some bound drug is released during the chasing period and [^{3}H]-

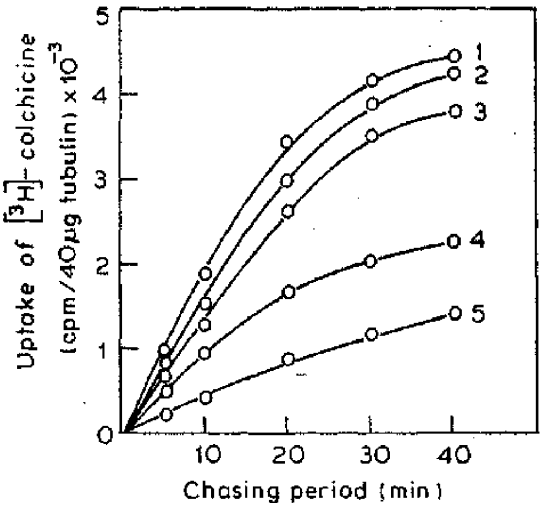

Figure 6. Binding of colchicine and its analogues to intact Zn(II)-induced polymers of tubulin. Aliquots of DEAE-tubulin (1·2 mg/ml) in bluffer A were assembled at 37°C for 30 min in the presence of 1×10^{-4} M $ZnSO_4$. Assembled mixtures were subsequently incubated with 1×10^{-4} M unlabelled drugs at 37°C for 15 min. Polymers were pelleted by warm (25°C) centrifugation at 120,000 *g* for 30 min. Pellets were then resuspended, depolymerized with 1×10^{-3} M EDTA and subsequently incubated with [^{3}H]-colchicine (1×10^{-6} M) at 37°C. The kinetics of uptake of [^{3}H]-colchicine was followed in each case by withdrawing suitable aliquots from the reaction mixture at different intervals and measuring the bound [^{3}H]-colchicine by DE-81 filter binding assay as described under 'materials and methods'.

(1), Control pellet obtained in the absence of any drug; (2), pellet obtained in the presence of colcemid; (3), pellet obtained in the presence of colchicine; (4), pellet obtained in the presence of 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone; (5), pellet obtained in the presence of desacetamido colchicine.

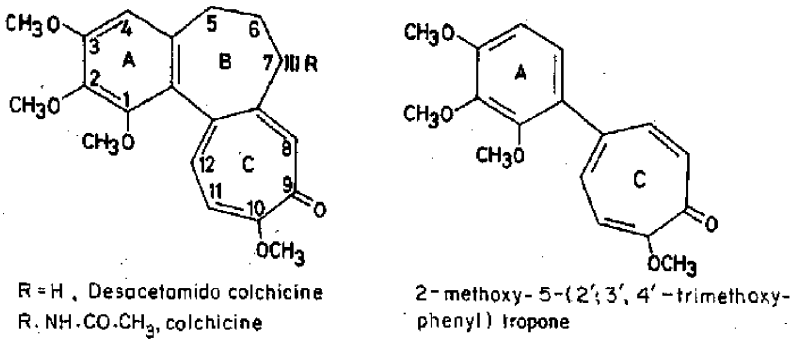

Figure 7. Structure of colchicine and its analogues.

colchicine gets bound to those vacant sites. These results clearly indicate that unlike colchicine and colcemid (both of which have a B-ring substituent), desacetamido colchicine (devoid of B-ring substituent), and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (devoid of B-ring) can bind to tubulin subunits in the intact Zn(II)-induced polymers.

Discussion

Although copolymerization of CD complex and tubulin dimer has been reported in normal microtubule assembly (Sternlicht and Ringel, 1979), the ratio of CD tubulin dimer in those microtubules is very low. It has been reported from our laboratory (Banerjee *et al*., 1982) that CD complexes could also be polymerized in the presence of $ZnSO_4$. The incorporation of colchicine into the polymer structure has been confirmed using [^{3}H]-colchicine. From this behaviour of colchicine, it is conceivable that the tubulin-tubulin interaction sites involved in the process of Zn(II)-induced assembly are different from the colchicine-binding site of tubulin. Thus, from the ability of colchicine-tubulin complex to form sheets in the presence of $ZnSO_4$ one might expect that the drug binding sites on tubulin subunits are exposed on the wall of Zn(II)-tubulin sheets. In order to test this possibility, the colchicine binding properties of the preformed Zn(II)-tubulin sheets have been studied. The results shown in figure 2 clearly indicate that the colchicine binding activity of tubulin is lost upon assembly in the presence of Zn(II). That the loss in the drug binding activity is not due to the inactivation or poisoning of tubulin is confirmed by the fact that preincubation of tubulin with Ca(II) or vinblastine sulphate or treatment of sheets with Zn(II)-chelators (to depolymerize them) could restore the drug binding activity. Moreover, the inhibition of the drug binding activity was not observed when tubulin was incubated with Zn(II) at a protein concentration far below the critical concentration for assembly in the presence of Zn(II). The above results indicate that the Zn(II)-tubulin sheets bind colchicine very poorly.

The assembly of tubulin at diferent Zn(II) concentrations was studied and it was found that inhibition of drug binding activity by Zn(II) was exhibited only when assembly was induced. Zn(II) at 1×10^{-5} neither induced assembly (figure 3) nor inhibited the colchicine binding activity of tubulin (figure 2). Moreover, the data obtained by the colchicine binding study correlated well with that obtained by the analysis of pellets after warm centrifugation (table 1).

Although the Zn(II)-induced assembly and normal microtubule assembly differ strikingly with respect to their sensitivity to colchicine, both polymers resemble each other with respect to colchicine binding properties *i.e*., neither of them can bind colchicine. Thus, the assurance of accommodating a drug molecule into a polymer structure does not necessarily mean that the polymer will bind the drug. One possible explanation for the inability of Zn(II)-induced polymers to bind colchicine might be that the alteration in conformation of the tubulin molecule which is required for colchicine binding (Garland, 1978; Lambier and Engelborgh, 1981) is hindered in the assembled state as compared to the free state. Infact, this hindrance is not unlikely for tubulin in the assembled state where each tubulin is anchored by adjacent tubulin molecules. An alternative mechanism might be that although the tubulin-tubulin interaction site is different from the colchicine binding site (at least in the case of Zn(II)-induced polymers), however, it is possible that in the assembled state, the colchicine binding site is partially covered (sterically crowded) by the adjacent tubulin molecule and thus prevents a good initial contact between the colchicine and the colchicine binding site of the tubulin molecule. To test these possibilities we have chosen the B-ring analogues of colchicine *viz*., desacetamido colchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone which bind to tubulin at the same site at which colchicine binds (Ray *et al*., 1981). The fluorometric results (figure 5)

clearly indicates that unlike colchicine, the binding of desacetamido colchicine and 2-methoxy-5-(2',3',4'–trimethoxyphenyl) tropone was almost the same in the case of tubulin either in the polymerized or in the unpolymerized state. Similar results have also been obtained in the chasing experiment (figure 6). Thus, it appeared from this study that the A- and C-ring (of colchicine) binding domains on tubulin are exposed in the Zn(II)-induced polymers. Even the compound with A-, C-, and B-ring (desacetamido colchicine) could bind to tubulin in the assembled state whereas the presence of -NH $CO.CH_3$ in the B-ring makes the compound inactive. Our experiments, however, do not distinguish whether this is purely a steric effects or due to a forbidden conformational change (as needed for colchicine binding) of tubulin in the assembled state. By what means the substituents on the B-ring of colchicine alter its binding properties with normal microtubules remain to be established.

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