B-ring of colchicine and its role in taxol-induced tubulin polymerization

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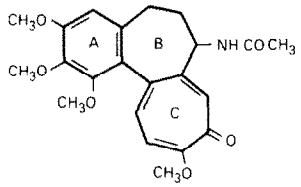
Taxol-induced assembly of purified tubulin is not inhibited by the colchicine analogue 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone. Colchicine analogues having intact A, C and B-rings (without NH-CO-CH_3) such as desacetamidocolchicine have also been found to be inactive. It has been observed that these two colchicine analogues are incorporated into polymers when incubated in the presence of taxol. Furthermore, preformed taxol-induced polymers of tubulin have been found to bind these two colchicine analogues. These results suggest that colchicine-binding domains on the tubulin molecule are mostly (if not completely) exposed in the taxol-induced polymers.

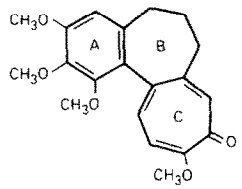
Taxol *Tubulin* *Polymerization* *Colchicine binding*

1. INTRODUCTION

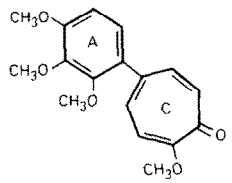
Colchicine and its analogues have been used extensively as a tool for the in vivo study of microtubule-dependent processes. In order to possess this colchicine-like activity, the analogue must have the trimethoxybenzene (A-ring) and methoxytropone (C-ring) systems combined into a single molecular entity by the appropriate carbon-carbon single bond [1,2]. Thus, the tetramethoxybicyclic compound 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (fig.1) possesses the minimal molecular features required for colchicine-like biological activity. This interesting analogue binds to tubulin at the same site as colchicine, inhibits in vitro microtubule assembly and is a potent mitotic inhibitor [1]. Here, we report that unlike normal microtubule assembly (MAPs-induced), taxol-induced tubulin assembly is not inhibited by 2-methoxy-5- (2',3',4'-trimethoxyphenyl)tropone. Colchicine analogues having intact A, C and B-rings (without NH-CO-CH_3) such as desacetamidocolchicine (fig.1) have also been found to be inactive. The results suggest that the B-ring substituent (NH-CO-CH_3) is important in distinguish-



COLCHICINE



DESACETAMIDO COLCHICINE



2 methoxy 5-(2',3',4' trimethoxyphenyl) tropone

Fig.1. Structure of colchicine and its analogues

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ing the protein-protein interaction sites involved in normal and taxol-induced polymerization.

2. MATERIALS AND METHODS

Colchicine, GTP (grade IIS), Mes (2-(*N*-morpholino)ethanesulfonic acid), bovine serum albumin (BSA) and SDS were products of Sigma. Phosphocellulose (P11) was obtained from Whatman I, England and Taxol from NCI, National Institutes of Health, USA. Desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone were the kind gifts of Dr Thomas J. Fitzgerald, School of Pharmacy, Florida A and M University, Tallahassee. All other chemicals were of analytical grade.

2.1. *Purification of tubulin*

Microtubule protein was isolated by two cycles of assembly-disassembly from goat brains following the procedure of Shelanski et al. [3]. The twice-cycled pellet was kept at −70°C. Tubulin was purified from this by phosphocellulose chromatography according to Weingarten et al. [4]. The tubulin thus obtained contains greater than 96% α- and β-tubulins, with the remaining 3–4% being distributed between 2–3 bands of lower M_r than tubulin, as determined by SDS gel electrophoresis [5]. The protein concentrations were determined as described by Lowry et al. [6], using BSA as a standard.

2.2. *Spectrophotometric assay of tubulin assembly*

Growth of microtubules was followed by turbidity measurement in assembly buffer (0.1 M Mes, pH 6.4; 0.5 mM $MgCl_2$; 1 mM GTP and 4 M glycerol) at 400 nm instead of 350 nm to avoid contributions from colchicine absorption. A Shimadzu UV 210A double-beam spectrophotometer was used. Temperature (37°C) was maintained in the cuvette chamber by an LKB 2209 Multitemp water circulator. Fluorescence was measured in a Perkin-Elmer MPF 44B fluorescence spectrophotometer. The temperature was controlled by the above water circulator.

3. RESULTS

The effect of different concentrations of colchicine on taxol-induced assembly of phosphocellulose purified goat brain tubulin is illustrated in fig.2. It is evident that colchicine inhibits assembly in a concentration-dependent manner and half-maximal inhibition occurs when only 13% of the total tubulin is complexed with colchicine. Bound tubulin concentrations are calculated from the apparent dissociation constant of colchicine with tubulin as 0.55 μM [7]. To our surprise, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone and desacetamidocolchicine have been found to be unable to inhibit taxol-induced assembly (fig.3.). Although the rate of polymerization is slightly decreased in the presence of each analogue, the final values of polymerization are almost unaffected. Polymers thus formed in the presence of analogues are cold-sensitive (fig.3). Nevertheless, these two analogues of colchicine are known to bind tubulin at the same site as colchicine and to inhibit in vivo and in vitro microtubule assembly [8–11]. It should be noted that this phosphocellulose-purified tubulin is unable to polymerize without taxol at such a low protein concentration.

This taxol-induced polymerization of tubulin in the presence of colchicine analogues (fig.3) indicates the incorporation of drug-tubulin complexes into polymers. To demonstrate the incorporation of analogue into polymers as tubulin-drug complex, we preincubated tubulin with 28 μM of either analogue at 37°C for 30 min to produce

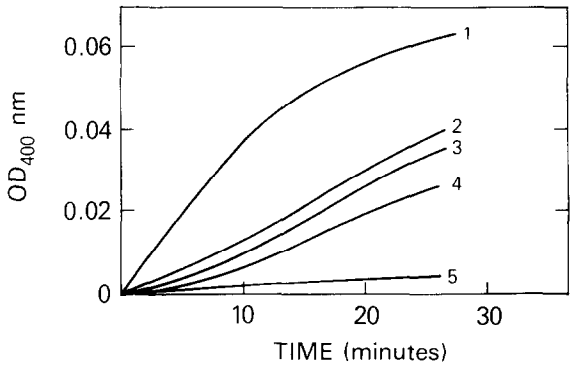

Fig.2. Inhibition of taxol-induced tubulin assembly by colchicine. 14μM tubulin was incubated with different concentrations of colchicine at 37°C for 90 min in assembly buffer. Polymerization was then initiated by the addition of taxol (10 μM) and assembly studied spectrophotometrically at 400 nm. Colchicine concentrations – curves: 1, 0; 2, 3 μM; 3, 4 μM; 4, 6 μM; 5, 9 μM.

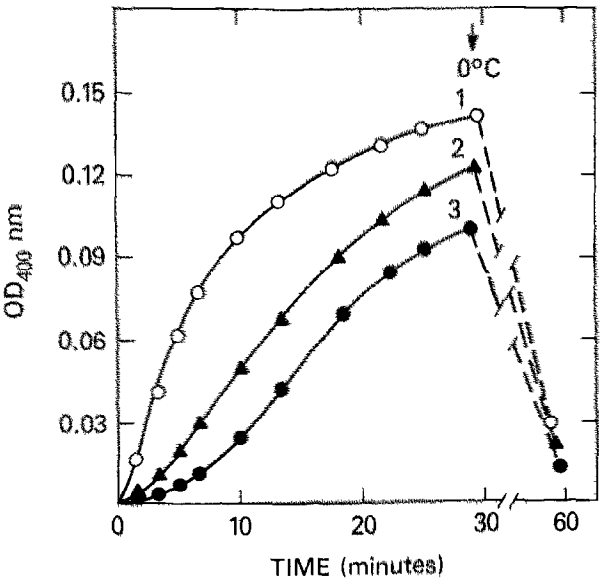

Fig.3. Taxol-induced assembly of tubulin in the presence of colchicine analogues. 14 μM tubulin in assembly buffer was polymerized by the addition of taxol (10 μM) at 37°C in the presence of colchicine analogues (10 μM). Polymerization was monitored spectrophotometrically at 400 nm. Samples: control (○—○); desacetamidocolchicine (▲—▲) and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (●—●).

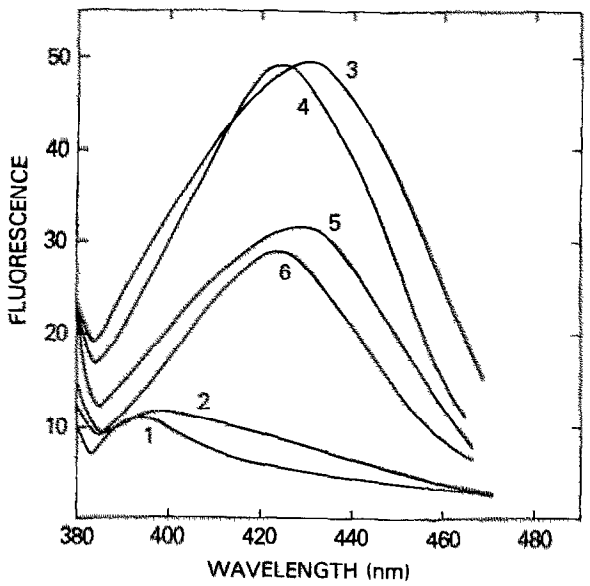

Fig.4. Incorporation of colchicine analogues into polymers assembled in the presence of taxol. Tubulin (14 μM) was incubated in assembly buffer with colchicine analogue (28 μM) at 37°C for 30 min to form drug-tubulin complex. An aliquot (1 ml) of drug-tubulin complex was polymerized in the presence of taxol (10 μM), polymers were pelleted by centrifugation at 100000×g for 30 min at 25°C and the pellet depolymerized in an equal volume (1 ml) of cold buffer. Fluorescence spectra of the samples were recorded as described in the text. (1) Tubulin only. (2) Disassembled polymer pellet assembled without drug. (3) Tubulin-desacetamidocolchicine complex before polymerization. (4) Tubulin-2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone complex before polymerization. (5) Disassembled polymer pellet assembled from tubulin-desacetamidocolchicine complex. (6) Disassembled polymer pellet assembled from tubulin-2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone complex. Tubulin concentrations of depolymerized samples were determined and found to be 13 μM in the absence of drug (curve 2), 10 μM in the presence of desacetamidocolchicine (curve 5) and 11 μM in the presence of 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (curve 6).

analogue-tubulin complex. Assembly was subsequently initiated by the addition of GTP and taxol. Polymers were then collected by warm centrifugation at 25°C. The pellet was suspended and depolymerized in cold buffer, subsequently being examined for fluorescence emission spectra after excitation at 350 nm. It is evident that the depolymerized products show fluorescence emission spectra characteristic of the respective drug-tubulin complex (fig.4). Incorporation of the analogue molecule into taxol-induced polymer indicates that the polymer could accommodate the drug molecule within its lattice.

Thus, the question may arise as to whether the preformed polymers bind these analogues. To study the binding of these analogues to taxol-induced polymers, tubulin was assembled to the steady state in the presence of taxol and subsequently incubated further with 10^{-4} M drug at 37°C for 30 min. The drug-treated polymers were pelleted by warm centrifugation at 25°C. The pellets were suspended in buffer, depolymerized by cold (0°C) and the fluorescence emission intensity at 430 nm determined for calculation of the amount of drug per mol tubulin. The stoichiometries were found to be 0.40, 0.43 and 0.002 for desacetamidocolchicine, 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone and colchicine, respectively.

4. DISCUSSION AND CONCLUSION

Assembly of microtubule protein in the presence of the GTP analogue pp(CH_2)pG is not inhibited by supra-stoichiometric concentrations of colchicine or its analogues [12]. Similar behavior of colchicine and its analogues was observed in the assembly of microtubule protein in the presence of zinc. These zinc-induced polymers of tubulin formed in the presence of colchicine contain an appreciable amount of incorporated colchicine and are cold sensitive [13]. The taxol-induced polymerization reported here is the first known which is inhibited by colchicine and not by its analogues. Although these two colchicine analogues (this paper) bind to tubulin at the same site as colchicine, nevertheless, there are important differences between colchicine and the two analogues. Thus, desacetamidocolchicine (having no substituent on the B-ring) and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone bind tubulin reversibly and the binding is instantaneous at 37°C [8,10,11] whereas colchicine binds tubulin very slowly and essentially irreversibly. Recently, significant differences in the binding and thermodynamic parameters such as activation energy, entropy and enthalpy have been reported for colchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone [11]. These differences in binding properties might be responsible for the different behavior of colchicine and its two analogues in taxol-induced tubulin assembly. Another possible mechanism might be that the colchicine-binding site on tubulin and the tubulin-tubulin interaction site on taxol-induced polymers share a common region on the tubulin molecule. Since colchicine and its structural analogue, desacetamidocolchicine, differ only in the side chain present on the B-ring, it is possible that the B-ring side chain binding region of tubulin is a common region which is also needed for the tubulin-tubulin interaction in the case of taxol-induced assembly. To gain better understanding of the relation between the B-ring of colchicine and the protein-protein interaction site involved in the polymerization process, the large number of interesting B-ring compounds synthesized by Capraro and Brosi [16] will be very helpful.

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