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Role of B-ring of Colchicine in Its Binding to Tubulin*

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The chemical specificity of the colchicine-binding site of tubulin is less stringent for the presence of the B-ring than the A- and C-rings of colchicine. Colchicine analogues with modifications in the B-ring bind to tubulin at the same site as colchicine. Analogues with smaller or no substituents in the B-ring bind tubulin remarkably faster than colchicine. Thus, a compound without the B-ring [2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone] binds tubulin even at 4 °C and the binding is almost instantaneous at 37 °C. Colcemid and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone bind reversibly to tubulin, whereas colchicine and desacetamidocolchicine bind almost irreversibly, suggesting that the size of the B-ring moiety of colchicine is not related to the reversibility of binding.

We conclude that although the presence of the B-ring of colchicine does not appear to be an essential prerequisite for the drug-tubulin interaction, the B-ring substituents play an important role in determining the binding properties of colchicine to tubulin.

Colchicine binds to native tubulin with a stoichiometry approaching 1 mol of alkaloid per mol of tubulin dimer (M_r = 110,000). Most of the published work involving binding of colchicine and its structural analogues to tubulin emphasize the importance of the role of the trimethoxyphenyl (A-ring) and tropolone (C-ring) moieties (1-3). Podophyllotoxin which resembles colchicine only in the A-ring inhibits colchicine binding to tubulin (1, 2, 4). Insertion of a bulky group in the A-ring of colchicine, as in colchicoside, causes complete loss of binding (1, 2). Similarly, the requirement of the tropolone ring (C-ring) is highly stringent. Thus, lumicolchicine which has an altered C-ring does not bind tubulin (4). Replacement of the tropolone by a phenyl ring produces the totally inactive drug colchinol (5). Even slight manipulation of the tropolone structure, by interchanging the positions of the carbonyl and methoxy groups, produces an inactive analogue, *i.e.* isocolchicine, having no tubulin-binding activity (1). On the other hand, colchicine analogues modified at, or depleted of, the B-ring are known to have potent antimitotic activity which apparently rules out any major role of the B-ring in the antimitotic activity of colchicine (3). Nevertheless, a minor change in the B-ring substituents may significantly change the binding mechanism and nature of the drug-tubulin complex formed as has been reported from this laboratory (6). Thus, colcemid binds tubulin fairly rapidly and reversibly, whereas colchicine binds slowly, requiring long incubation at 37 °C to attain equilibrium (7-10) and the binding is reversible with difficulty (4, 11). This finding prompted us to investigate whether the B-ring and its substituents have any role on the rate and reversibility of the reaction.

It was found on the basis of the present study, using colchicine analogues differing in the B-ring only, that the smaller the substituents are on the B-ring, the faster is the rate of binding of the analogue to tubulin. Moreover, unlike colchicine, the analogues with modified B-rings bind tubulin even at 4 °C.

MATERIALS AND METHODS

Tubulin was purified from goat brain in PMG buffer (10 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 0.1 mM GTP) by the procedure of Weisenberg *et al.* (12), except that DEAE-cellulose was used instead of DEAE-Sephadex. The active fractions, as judged from a colchicine binding assay, were pooled, concentrated by overnight dialysis at 0 °C against 8 M glycerol in either binding buffer (PMG) or polymerization buffer (100 mM Mes[1] pH 6.4, 1.0 mM EGTA, 1.0 mM GTP, and 0.5 mM $MgCl_2$) depending on the nature of subsequent experiments, and stored at −20 °C. The purity of tubulin was checked by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate according to Weber and Osborn (13).

Protein concentrations were determined as described by Lowry *et al.* (14), using bovine serum albumin as a standard. The GF/C filter paper disc assay method described by Banerjee and Bhattacharyya (6) for colcemid and colchicine binding was used with a minor modification for monitoring the effect of the unlabeled analogues (which interact with tubulin either reversibly or irreversibly) on [^{3}H]colchicine-binding to tubulin. Whatman GF/C filter paper discs were initially washed with 1 ml of cold (2-4 °C) PM buffer (10 mM potassium phosphate, pH 7.0, and 10 mM $MgCl_2$), taking care not to dry the papers. Then, 1 ml of PM buffer containing 10^{-5} M colchicine was poured on the filter paper to which 100 μl of the sample was added and allowed to drain off under mild suction. The filters were then rinsed 3 times with 3 ml of cold PM buffer by mild suction, dried, and counted in 5 ml of toluene-based fluor. Identical blanks, without tubulin, were prepared and necessary corrections were made.

Fluorescence was measured as described by Bhattacharyya and Wolff (1) in a thermostated chamber at 37 °C in a Perkin-Elmer MPF 44B spectrofluorometer with an excitation wavelength of 353 nm. Microtubule assembly was measured spectrophotometrically in polymerization buffer containing 4 M glycerol at 37 °C in a Cary 17D spectrophotometer recording at 400 nm. The polymerization was initiated by raising the temperature of the reaction mixture from 0-37 °C directly in the thermostated chamber of the spectrophotometer.

Tritiated colchicine (ring C, [^{3}H]methoxy), having a specific activity of 5 Ci/mmol, was the product of New England Nuclear Corp. Colcemid was a gift from Dr. J. Wolff of the National Institutes of Health. The other colchicine analogues, desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone were kindly supplied by Dr. T. J. Fitzgerald of Florida A and M University. Unlabeled colchicine and GTP (grade IIS) were products of Sigma Chemical Co. GF/C filter papers were obtained from Whatman Ltd., England.

RESULTS AND DISCUSSION

B-ring Specificity of Colchicine Binding Site

To elucidate the role of the B-ring of colchicine in its binding to tubulin, structural analogues were chosen which

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[1] The abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

differ from colchicine in the B-ring only (Fig. 1). Among these compounds, colchicine and colcemid have been widely used in the study of the tubulin-microtubule system. Analogues such as desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone are reported (3) to inhibit tubulin polymerization, but their effects on the colchicine binding site of tubulin have not been tested. The ability of all these drugs to inhibit microtubule assembly both *in vitro* and *in vivo* apparently rules out any major role of the B-ring of colchicine in its antimicrotubular activity (3). Nevertheless, these findings do not furnish any information as to whether and how the B-ring of colchicine affects the mechanism of colchicine-tubulin interaction. Thus, it was important to examine first whether all the chosen compounds bind to tubulin at the same site as colchicine. The B-ring specificity of colchicine-tubulin interaction was studied by measuring the prevention of [^{3}H]colchicine binding by the analogues with modified B-ring (Fig. 1) and the data were analyzed by modified Dixon plots (Fig. 2). From the results, it is evident that [^{3}H]colchicine binding is inhibited competitively by colcemid, desacetamidocolchicine, and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone yielding apparent K_i values of 1.4×10^{-5} M, 0.32×10^{-5} M, and 1.6×10^{-5} M, respectively.

Thus, it appears from this study that, unlike the A-ring and the C-ring, a wide variation in the B-ring moiety is tolerated by the colchicine binding site of tubulin. Even 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone, which is devoid of the B-ring of colchicine, showed a considerable inhibition of colchicine binding. This suggests that the site of interaction of colchicine on tubulin is embodied in the bicyclic derivative, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone.

Role of the B-ring Moiety on the Rate of Colchicine-Tubulin Interaction

Colchicine binding to tubulin is a slow process which needs long incubation at 37 °C for equilibration (7-11). There is no direct evidence to explain this unusual property, although it has been interpreted as showing the requirement for a favorable conformation of tubulin molecule for binding (15) or a colchicine-induced change in its conformation (16). However, it has not been settled unequivocally whether the slow binding is a property of the binding site of tubulin which needs long incubation at 37 °C for conformational change to occur or is due to the unfavorable conformation of the colchicine molecule. We therefore examined these possibilities using the B-ring-modified colchicine analogues.

It is already known that although neither colchicine nor tubulin exhibit any fluorescence in PMG buffer (pH 7.0) when excited at the absorption peak of colchicine, there is striking induction of fluorescence when these two compounds are incubated together at 37 °C (1). So it was thought appropriate to examine the fluorescence property of the colchicine analogues which interact with tubulin. It was found, using a solution containing tubulin and 5 μM drug in PMG buffer (pH 7.0), that colcemid, desacetamidocolchicine, and 2-methoxy-5-

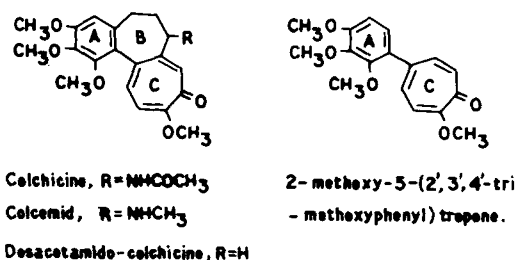

FIG. 1. **Structure of colchicine and some of its analogues.**

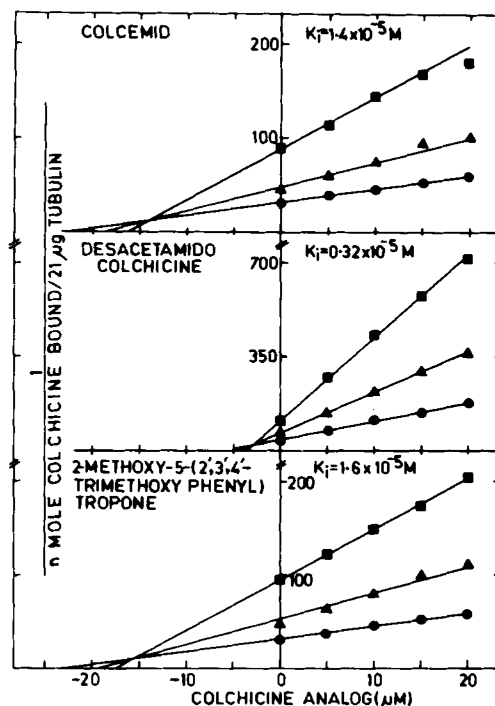

FIG. 2. **Modified Dixon plot demonstrating the effect of colchicine analogues on the colchicine binding to tubulin.** The concentrations of the analogues present is plotted against the reciprocal of the bound [^{3}H]colchicine at several concentrations of [^{3}H]colchicine: (■——■) 1 μM; (▲——▲) 2 μM; (●——●) 4 μM. The reaction mixtures containing tubulin (0.21 mg/ml) and the drugs as indicated were incubated at 37 °C for 90 min.

(2′,3′,4′-trimethoxyphenyl)tropone fluoresce on combination with tubulin, with the emission maxima in the same region as in the case of colchicine. However, fluorescence of the colcemid-tubulin complex had a comparatively low quantum yield.[2] Furthermore, like colchicine, the other compounds did not fluoresce in the absence of tubulin. So, it was convenient to measure the time of equilibration of binding of these ligands to tubulin by this method. Tubulin was mixed with 5 μM drug at 37 °C in a temperature-controlled spectrofluorometer, and the emission spectra were measured at different time intervals. It is evident from Fig. 3 that, while the binding of colchicine was not complete even within 75 min, colcemid binding to tubulin was complete in about 20 min. A similar result was also obtained for colcemid by monitoring binding of the ^{3}H-labeled drug to tubulin.[2] In the case of desacetamidocolchicine, maximum fluorescence, indicating maximum binding, was obtained in about 15 min. Interestingly, binding of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (a compound which is devoid of the B-ring) to tubulin was very rapid since within 1.5 min maximum fluorescence was obtained which did not increase further with time.

From the fluorescence data it is evident that depletion of substituents from the B-ring of colchicine molecule causes concomitant enhancement in the rate of its binding to tubulin and reduction in the time of equilibration. Thus, the question arises as to whether the slow binding of colchicine is due to its extreme structural rigidity, since a compound (2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone), which can assume an infinity of conformations due to free rotation around the single bond, binds tubulin almost instantaneously. However, this seems unlikely, because the rigidity imparted to colchicine molecule by the B-ring, which anchors the A- and C-rings

[2] K. Ray, B. Bhattacharyya, and B. B. Biswas, unpublished observation.

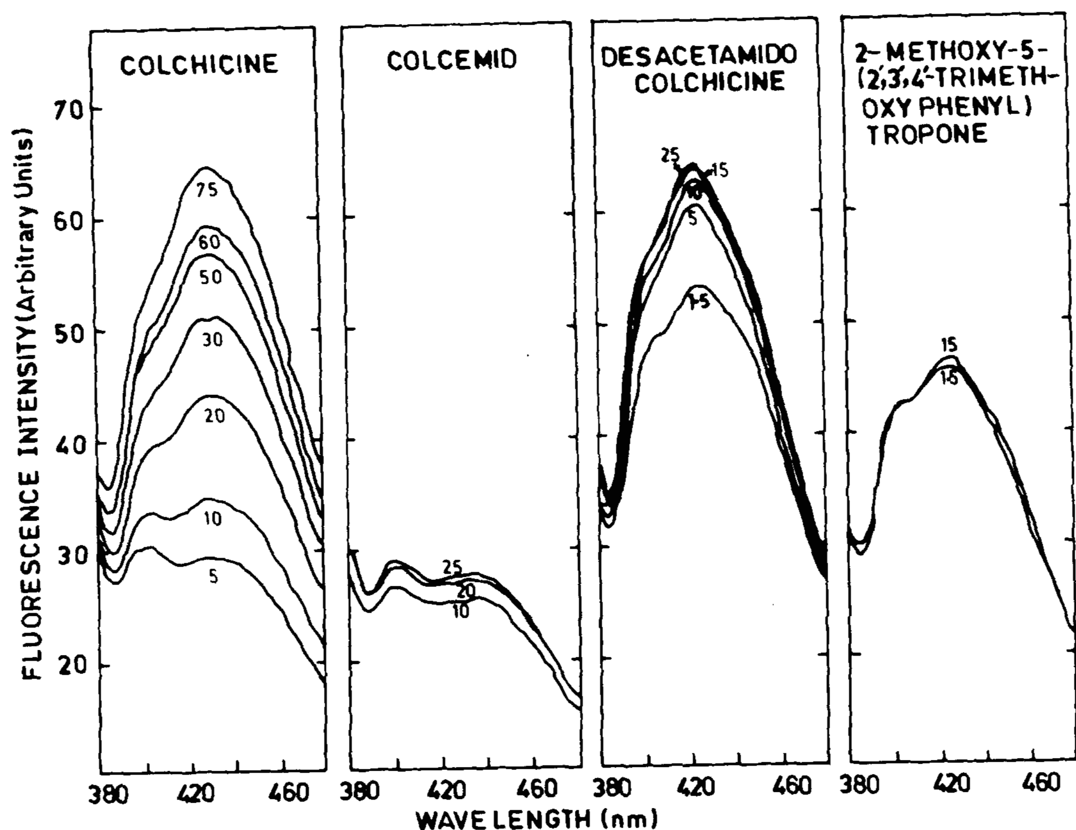

FIG. 3. **Time dependence of fluorescence emission spectrum of the drug-tubulin complex.** The reaction mixture contained 9.4 μM tubulin and 5 μM drug in PMG buffer (pH 7.0). The *numbers* accompanying each *curve* refer to time (in minutes) of incubation of the reaction mixture at 37 °C. The excitation wavelength was 353 nm.

TABLE I

The effect of temperature on binding of colchicine analogues to tubulin

The reaction mixtures containing 10 μM tubulin and 5 μM drug were incubated in PMG buffer (pH 7.0) at 37 °C or 4 °C for 60 min. Fluorescence intensity was measured at 430 nm and proper corrections were made for blank (minus tubulin). The excitation wavelength was 353 nm.

Colchicine analogues	Condition	Per cent of maximal fluorescence
Colchicine	37 °C	100
	4 °C	9
Colcemid	37 °C	100
	4 °C	Inconclusive
Desacetamidocolchicine	37 °C	100
	4 °C	73
2-Methoxy-5-(2',3',4'-trimethoxyphenyl)tropone	37 °C	100
	4 °C	100

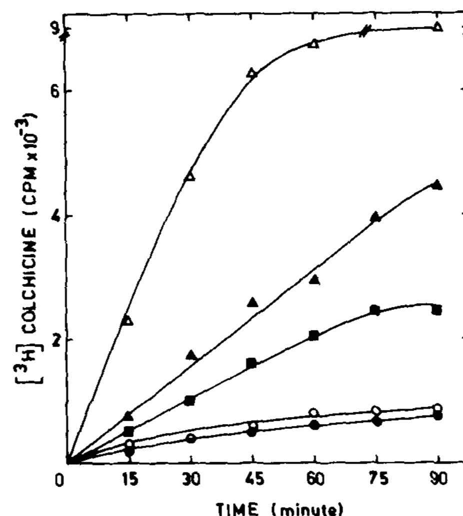

FIG. 4. **Reversibility of binding of colchicine analogues to tubulin.** Tubulin (3.6 μM) in PMG buffer (pH 7.0) was preincubated at 37 °C for 45 min in the absence (△——△) or presence of colchicine analogues (12.5 μM): colcemid (▲——▲), 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (■——■), desacetamidocolchicine (○——○), colchicine (●——●). The assay mixture containing only tubulin (control tube) or drug-tubulin complex was further incubated with [^{3}H]colchicine (1 μM) at 37 °C for different times as indicated in the figure. Tubulin bound radioactivity was determined by GF/C filter paper disc assay as described under "Materials and Methods."

together, is present also in colcemid and desacetamidocolchicine. Rather, it is possible that gradual depletion of substituents from the B-ring results in the formation of analogues of favorable conformation for binding. In fact, the configuration of the B-ring, when present, is important as evidenced by the remarkable potency difference between the (+)-(unnatural) and (−)-enantiomers of colchicine for both tubulin (17) and the anti-colchicine antibody (18).

Next, we tested whether any of these colchicine analogues bind to tubulin at 4 °C where no conformational change of tubulin was expected to occur. So, 5 μM drug was mixed with 10 μM tubulin and exposed for 60 min either at 37 °C or at 4 °C. The fluorescence due to the drug-tubulin complex was measured. It is evident from Table I that in the case of colchicine, there was very little fluorescence in the sample incubated at 4 °C, whereas desacetamidocolchicine binding at 4 °C was about 73% of that at 37 °C. Interestingly, 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone binding to tubulin was complete at 4 °C and had exactly the same value as at 37 °C. However, nothing conclusive was found in the case of colcemid

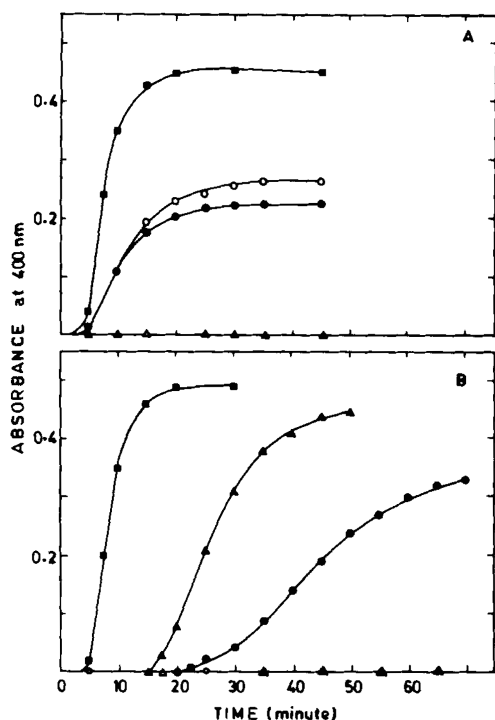

FIG. 5. **Reversibility of inhibition of tubulin polymerization by colchicine analogues.** Tubulin (2.6 mg/ml) in 4 M glycerol was allowed to polymerize at 37 °C in a buffer containing 100 mM Mes (pH 6.4), 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP: *A*, in the absence of drugs (■——■) or in the presence of 10 μM drug (colchicine (○——○), colcemid (●——●), desacetamidocolchicine (△——△), 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (▲——▲)). After monitoring polymerization at 400 nm by incubating the reaction mixture at 37 °C for 45 min, the microtubules formed were depolymerized by exposing the tubes containing the reaction mixture at 0 °C for 10 min. Then 20 mg of charcoal (previously washed with 100 mM Mes (pH 6.4), 1 mM EGTA, and 0.2% bovine serum albumin) was added per tube, kept for 30 min at 0 °C with occasional stirring, and then spun to pellet the charcoal. *B*, the charcoal-free reaction mixtures were again incubated at 37 °C to monitor polymerization of tubulin.

because of the weak fluorescence of the tubulin-colcemid complex. These observations seem to exclude the possibility of slow conformational change of tubulin as an essential prerequisite for its binding to the analogues like desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone. So, it seems probable that the slow binding of colchicine to tubulin, which is reported to occur with accompanying change in tubulin conformation (16), might be due to an unfavorable conformation of the colchicine molecule. Recently, Detrich *et al.* (19) have shown that the circular dichroic spectrum of colchicine changes on binding to tubulin, indicating that a conformational change of colchicine occurs on binding. Wolff *et al.* (18) have observed that, in contrast to the slow binding of colchicine to tubulin even at 37 °C, the drug binds to its antibody readily at 4 °C.

Reversibility

Unlike colchicine binding, colcemid binding to tubulin is reversible (6). This finding suggests that, in addition to its effect on the rate of colchicine-tubulin interaction, the B-ring of colchicine might play a significant part in the nature of the drug-tubulin complex formation. To explore this possibility, the colchicine analogues were tested for their reversibility of interaction with tubulin.

By Binding Experiments—The extent of reversibility of interaction of the analogues with tubulin were quantitated by chasing the preformed analogue-tubulin complex with $[^3H]$-colchicine. For this, tubulin was preincubated for a fixed time with the analogues (12.5 μM) to be tested and then chased with $[^3H]$colchicine (1 μM) for different time intervals by further incubation at 37 °C. The rate of formation of the $[^3H]$colchicine-tubulin complex was taken as a measure of reversibility of interaction between the analogue and tubulin. As shown in Fig. 4, it appears that the interaction of tubulin with colcemid is maximally reversible, followed by the compound with the open B-ring, *i.e.* 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone. Desacetamidocolchicine is as irreversible as colchicine.

By Polymerization—Tubulin was allowed to polymerize both in the presence and absence of the drugs. It was thus found that 10 μM colchicine and colcemid inhibited polymerization partially in buffer containing 4 M glycerol while desacetamidocolchicine and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone inhibited polymerization of tubulin completely (Fig. 5). Such an observation can be explained by the faster rate of binding of the latter two drugs. On removal of the free drugs from the assay mixture by bovine serum albumin coated charcoal, after depolymerization (at 0 °C) of microtubule formed, it was found that tubulin treated with colcemid and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone only could again polymerize, indicating reversibility of interaction. These results agree with the K_i values of the drugs (Fig. 2). However, on the basis of the results presented here, it is apparent that the size of the B-ring moiety of colchicine cannot be related to the reversibility of interaction.

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