Eur. J. Biochem. *142*, 577–581 (1984)

Anion-induced increases in the affinity of colcemid binding to tubulin

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(Received February 29, 1984) – EJB 84 0226

Colcemid binds tubulin rapidly and reversibly in contrast to colchicine which binds tubulin relatively slowly and essentially irreversibly. At 37 °C the association rate constant for colcemid binding is 1.88×10^6 M^{-1} h^{-1}, about 10 times higher than that for colchicine; this is reflected in the activation energies for binding which are 51.4 kJ/mol for colcemid and 84.8 kJ/mol for colchicine. Scatchard analysis indicates two binding sites on tubulin having different affinities for colcemid. The high-affinity site ($K_a = 0.7 \times 10^5$ M^{-1} at 37 °C) is sensitive to temperature and binds both colchicine and colcemid and hence they are mutually competitive inhibitors. The low-affinity site ($K_b = 1.2 \times 10^4$ M^{-1}) is rather insensitive to temperature and binds only colcemid. Like colchicine, 0.6 mol of colcemid are bound/mol of tubulin dimer (at the high-affinity site) and the reaction is entropy driven (163 J K^{-1} mol^{-1}).

Similar to colchicine, colcemid binding to tubulin is stimulated by certain anions (viz. sulfate and tartrate) but by a different mechanism. Colcemid binding affinity at the lower-affinity site of tubulin is increased in the presence of ammonium sulfate. Interestingly, the lower-affinity site on tubulin for colcemid, even when converted to higher affinity in presence of ammonium sulfate, is not recognized by colchicine.

We conclude that tubulin possesses two binding sites, one of which specifically recognized the groups present on the B-ring of colchicine molecule and is effected by the ammonium sulfate, whereas the higher-affinity site, which could accommodate both colchicine and colcemid, possibly recognized the A and C ring of colchicine.

Colcemid (deacetyl-*N*-methyl colchicine), a structural analogue of colchicine with slightly modified B-ring, is also used as a microtubule inhibitor. Both the drugs interfere with microtubule-dependent cell functions because they bind tubulin, the subunit of microtubule [1–6]. Nevertheless, these two antimitotic drugs have remarkable differences in biological functions. Thus, the growth of yeast cells could be arrested by colcemid and not by colchicine [7]. Studies on mitotic arrest of different cells in culture with colchicine and colcemid suggest a more rapid release of bound colcemid than that of colchicine [8]. This property of colcemid make it a superior mitotic inhibitor than colchicine in the tissue culture cells. Finally, a preliminary report from this laboratory indicated that colcemid binds tubulin fairly rapidly and reversibly, whereas colchicine binds slowly, requiring a long incubation at 37 °C to attain equilibrium and the binding is reversible with difficulty [9]. These differences in properties between colchicine and colcemid made it difficult to envisage a mechanism for the apparent competition between these two ligands for the same binding site on tubulin and thus one of our interests was to compare some of the kinetic and thermodynamic parameters for the binding of these two compounds.

There is another aspect of colcemid-tubulin interactions in which we are interested. The importance of B-ring of colchicine in its binding to tubulin was emphasized recently by this laboratory [9,10]. It was found that two important unusual properties of the colchicine-tubulin interaction, such as the slow association rate (which needs a long incubation at 37 °C) and the high temperature coefficient, are phenomena of the B-ring substituents of colchicine. Thus, colchicine analogues with smaller (colcemid) or no substituents in the B-ring (desacetamido colchicine) bind tubulin remarkably faster than colchicine. A compound without the B-ring [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone] binds tubulin at 4 °C and the binding is almost instantaneous at 37 °C.

However, it was reported earlier that the rate of colchicine binding to tubulin could be enhanced by anions like tartrate and sulfate [11,12]. Although it is not known whether those anions bind tubulin and thereby modify tubulin in such a way that the B-ring binding domain of tubulin becomes easily accessible to the ligand, we thought it would be interesting to see whether those anions further enhanced the rate of colcemid binding to tubulin.

MATERIALS AND METHODS

Materials

Both [^{3}H]colchicine and [^{3}H]colcemid (ring C, [^{3}H]methoxy) having specific activities of 5 Ci/mmol and 9.3 Ci/mmol respectively were products of New England Nuclear Crop. GTP (grade IIS) and colchicine were products of Sigma. Colcemid was obtained from K and K. GF/C and DE 81 filter papers were products of Whatman. All other chemicals used were reagent grade.

Methods

Tubulin was purified from goat brain in Pi/$MgCl_2$/GTP buffer (10 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 0.1 mM GTP) by the procedure of Weisenberg et al. [2] 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 Pi/$MgCl_2$/GTP buffer and stored at −70 °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 [9] for colcemid and colchicine binding was used with minor modifications. The Whatman GF/C filter paper discs were initially washed with 1 ml of cold (2–4 °C) $Pi/MgCl_2$ buffer (10 mM potassium phosphate, pH 7.0 and 10 mM $MgCl_2$), taking care not to dry the papers. Then, 1 ml of $Pi/MgCl_2$ buffer containing 10 μM drug 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 three times with 3 ml of cold $Pi/MgCl_2$ buffer by mild suction, dried and counted in 5 ml of toluene-based fluor. Identical blanks, without tubulin, were prepared and the necessary corrections were made.

RESULTS AND DISCUSSION

Time of equilibration and the reversibility

Fig. 1 confirmed our previous report [9] that the binding of colchicine (1 μM) to tubulin required about 2 h to equilibrate at 37 °C (Fig. 1A) whereas the binding of colcemid (1 μM) occurred at a much faster rate requiring about 45 min to reach equilibrium (Big. 1B). It is further confirmed that while colchicine binding to tubulin is essentially irreversible, colcemid binding to tubulin is highly reversible. In the same experiment at definite intervals, aliquots containing tubulin-[^{3}H]drug complexes were withdrawn, mixed with 200-fold excess unlabelled drug and incubated further at 37 °C; the binding was then assayed at different time intervals. Any loss of protein-bound radioactivity upon the addition of the unlabelled ligand reflected the displacement of the initially bound labelled ligand and hence the reversibility of the interaction.

Thus, when an excess of unlabelled colcemid (200 μM) was added to tubulin that had been preincubated with [^{3}H]colcemid (1 μM) and was incubated further, about 80% (at maximum) of the initially bound radioactivity was displaced within 1 h (Fig. 1A). In contrast, the initial radioactivity bound to tubulin in a parallel experiment with [^{3}H]colchicine (1 μM) remained largely unaffected upon the addition of 200 μM unlabelled colchicine (Fig. 1B). This explained the behaviour of colcemid *in vivo* in the tissue culture [8].

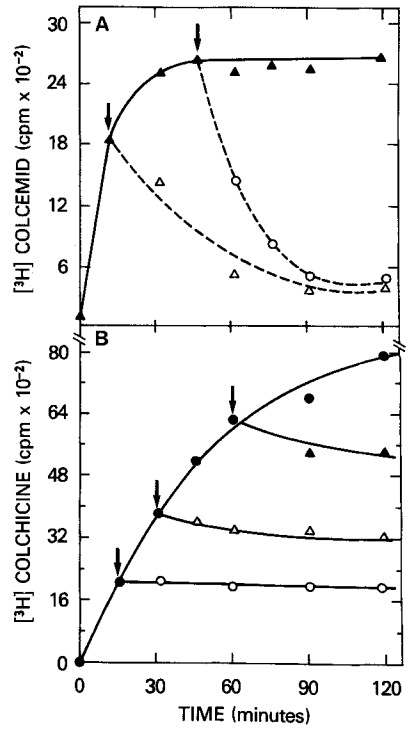

Fig. 1. *Reversibility of binding of colcemid and colchicine to tubulin.* Tubulin (2.9 μM) and 1 μM [^{3}H]colchicine or [^{3}H]colcemid were incubated together at 37 °C for different time. At indicated periods appropriate amount of unlabelled drug was added to the respective reaction mixtures to make final concentration of the drug 200 μM. The reation mixtures were incubated further at 37 °C, aliquots were withdrawn at different intervals and assayed for bound radioactivity. (A) Binding of 1 μM [^{3}H]colcemid (▲) and the effect of addition of 200 μM unlabelled colcemid at 10 min (△) or 45 min (○). (B) Similarly, binding of 1 μM [^{3}H]colchicine (●) and the effect of addition of 200 μM unlabelled colchicine at 15 min (○) or 30 min (△) or 60 min (▲)

Association rate constant

Assuming that binding of tubulin to colcemid is a bimolecular reaction, the association rate constant (k_1) can be represented as:

$$k_1 = \frac{d[CT]}{dt} / [C][T] \qquad (1)$$

where $\frac{d[CT]}{dt}$ is the rate of formation of the complex CT (colcemid-tubulin) and [C] and [T] are the concentrations of free colcemid and unbound tubulin respectively. Conditions were adjusted such that < 10% of the reactants were consumed during the intervals when the curves were linear and we have thus assumed that $[C]=[C]_0$ and $[T]=[T]_0$, where $[T]_0$ and $[C]_0$ are the initial concentrations of tubulin and colcemid respectively.

In one set of experiments, tubulin concentrations were held constant (195 nM) and the rate curves were linear for about 5 min over a concentration of 100–200 nM colcemid (Fig. 2A). In the other set, when colcemid concentration was 140 nM, the curve showed linearity for only 2 min over tubulin concentrations of 146–293 nM (Fig. 2B). The mean value of the second-order rate constants (k_1) has been calculated from these data, and was found to be 1.88×10^6 M^{-1} h^{-1}. The rate constant for colchicine is reported to be $0.12-0.48 \times 10^6$ M^{-1} h^{-1} [11,15,16]. Interestingly it was observed that the bulkiness of the substituents at the B-ring of colchicine analogues was important in determining the rate of association of the colchicine analogues to tubulin [10]: the smaller the substituent on the B-ring, the faster the reaction. In the case of colcemid the activation energy for its assiciation with tubulin was found to be 51.4 kJ/mol (12.3 kcal/mol, unpublished observation) whereas that for colchicine is reported to be 84.8 kJ/mol (20.3 kcal/mol) [17]. So comparatively faster binding of colcemid as also reflected by the difference in association rate constants of the two drugs may be partly explained by the lower activation energy for colcemid binding.

Binding parameters at equilibrium

If the colcemid-tubulin binding reaction is:

$$T + nC = TC_n \qquad (2)$$

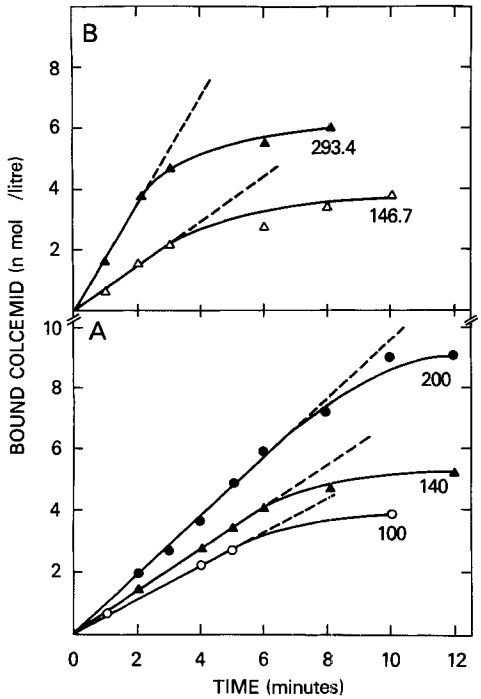

Fig. 2. *The rate of colcemid binding as a function of concentrations of colcemid and tubulin.* All incubations were done at 37 °C in Pi/$MgCl_2$/GTP buffer. (B) Colcemid was constant at 140 nM. Numbers refer to tubulin concentration (nM). (A) Tubulin was held constant at 195.6 nM. Numbers refer to colcemid concentration (nM). The dashed lines are continuations of the slopes for the initial rates.

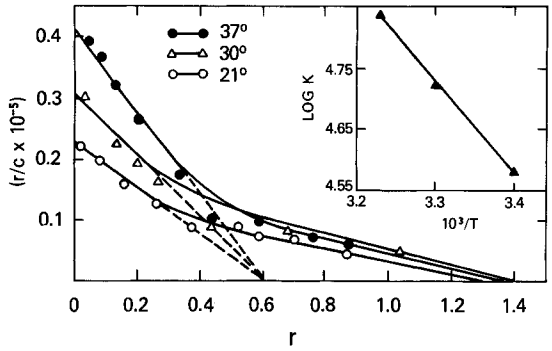

Fig. 3. *Scatchard plot of colcemid binding to tubulin at different temperatures.* The reaction mixtures containing tubulin (2.3 μM) and increasing concentrations of [^{3}H]colcemid were incubated at 21 °C (○) or 30 °C (△) or 37 °C (●) for 60 min. The assay was performed for tubulin-bound radioactivity. Free [^{3}H]colcemid concentrations were determined from the difference between the total and bound ligand. r = molar ratio of colcemid bound/tubulin, c = colcemid concentration. The inset shows log K against $1/T$ (K^{-1})

where T is tubulin, C is colcemid and n is the number of colcemid binding sites on tubulin, then at equilibrium:

$$r = nK[C]/(1 + K[C]) \quad (3)$$

were r is the molar ratio of colcemid bound/tubulin, K is the affinity constant:

$$K = [TC_n]/[C]^n[T]. \quad (4)$$

Rearrangement of Equation (3) gives:

$$r/[C] = nK - Kr. \quad (5)$$

The affinity of colcemid for purified goat brain tubulin was determined by analysing binding values obtained over a wide range of colcemid concentrations by the method of Scatchard as shown in Fig. 3. In contrast to colchicine binding, analysis of colcemid binding to tubulin yielded a non-linear Scatchard plot with a concavity upward. This suggested the presence of two or more classes of binding sites with different affinities for colcemid and the results were analyzed on the assumption of two non-interacting sites. Such treatment of the data yielded a high-affinity site with an affinity constant, K_a of 0.7×10^5 M^{-1} at 37 °C; 0.6 mol colcemid was bound to this site/mol tubulin dimer (M_r 110000). Also present in tubulin was a second and lower-affinity site, which binds colcemid with an affinity constant, K_b of 1.2×10^4 M^{-1} and a stoichiometry of 1.3 mol total colcemid bound/tubulin. This value of stoichiometry is significantly lower than that which is expected for 1:1 stoichiometry for each site. However, a parallel experiment carried out with colchicine has also furnished an indentical stoichiometry of 0.6 mol colchicine/mol tubulin dimer (by the GF/C paper assay). Furthermore, the low-affinity site was found to be almost insensitive to temperature.

Table 1. *Effect of anions on the colcemid binding activity of tubulin* The samples contained 3.2 μM tubulin, 1 μM [^{3}H]colcemid and 0.96 M salt. They were incubated at 37 °C for 5 min. r_A/r_0 represents ratio of tubulin-bound radioactivity in the presence (r_A) and absence (r_0) of salt

Salt	r_A/r_0
$(NH_4)_2SO_4$	2.20
Na_2SO_4	3.04
Na-K *meso*-tartrate	2.72
NH_4Cl	0.84
NaCl	1.12

Since the low-affinity site is independent of temperature, for the determination of thermodynamic parameters of interaction of tubulin with colcemid, the high-affinity site has been taken into consideration. A Van't Hoff plot of log K versus the reciprocal of the absolute temperature was linear in the region 21 – 37 °C (inset Fig. 3). Thus, the value of standard enthalpy of binding ($\Delta H°$) calculated from the slope of the curve was 21.9 kJ/mol (5.24 kcal/mol). The standard free energy ($\Delta G°$) of binding at 37 °C was equal to −28.7 kJ/mol (−6.87 kcal/mol) and $\Delta S° = 163$ J (39 cal) K^{-1} mol^{-1}.

Effects of anions on binding of colcemid to tubulin

Colcemid binding of dialyzed and undialyzed tubulin, preserved as a 50% ammonium sulfate precipitate, has significant differences in binding activity which could not be explained by simple decay during the period of dialysis and appeared to be associated with the presence of ammonium sulfate. Like anion-induced enhancement of colchicine binding to tubulin [11,12], this increased binding is only observed when the anion was either sulfate or tartrate. Cations such as NH_4^+ or Na^+ did not effect the binding (Table 1). A significant difference is observed between anion-induced colchicine and colcemid binding to tubulin. In the case of colchicine, this enhanced binding is observed at relatively low concentrations of anions, whereas at the same concentrations, colcemid binding to tubulin in almost unaffected. However, with the increase of anion concentration there is a significant increase in

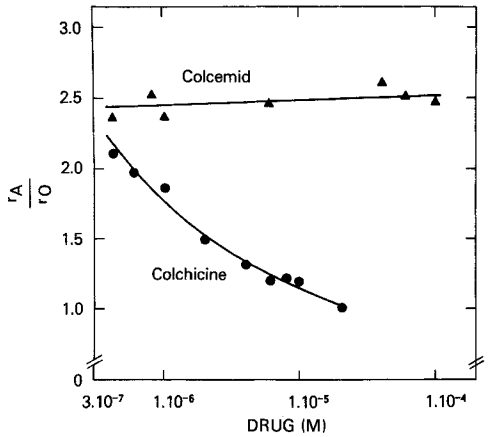

Fig. 4. *Dependency of ammonium-sulfate-induced rate enhancement of tubulin binding to colchicine or colcemid on the concentration of the respective ligand.* The rate was measured after a 6-min incubation at 37 °C with (r_A) and without (r_0) 1.6 M $(NH_4)_2SO_4$. The tubulin was 3.18 μM in all cases with various concentrations of [^{3}H]colchicine (●) or [^{3}H]colcemid (▲)

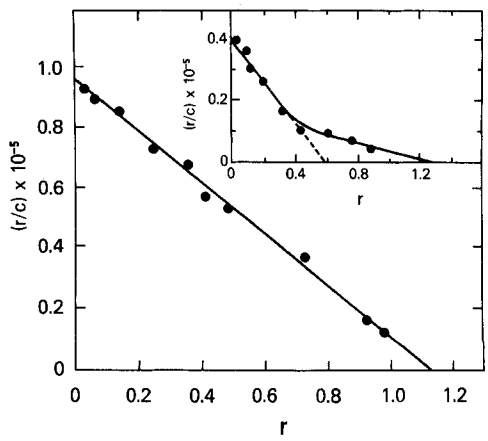

Fig. 5. *Scatchard plot of colcemid binding to tubulin in the presence of ammonium sulfate.* The reaction mixture containing tubulin (2.35 μM), $(NH_4)_2SO_4$ (1.6 M) and increasing concentrations of [^{3}H]colcemid was incubated at 37 °C for 60 min. Bound radioactivity was assayed and the free colcemid concentrations were determined from the difference between total and bound ligand. The inset shows a plot without $(NH_4)_2SO_4$, data taken from Fig. 3

colcemid binding to tubulin which approaches a plateau at arround 1.6 M ammonium sulfate. Neither NaCl nor NH_4Cl at that concentration affected binding. This makes it unlikely that the enhanced binding is due simply to ionic strenght.

Nature of the anion effect

Unlike anion-induced increases in the rate of colchicine binding [11], the enhancement in the colcemid binding to tubulin in the presence of anions appeared not to be due to an increase in the rate of the reaction. If the stimulation is truly mediated by enhancing the rate of binding, then it is expected that at higher ligand concentration where the rate of binding will be naturally faster [18,19], the stimulatory effect of $(NH_4)_2SO_4$ would be less effective. Typical experiments relating the $(NH_4)_2SO_4$ effect and the drug concentrations [ratio of binding rate with $(NH_4)_2SO_4$ to the rate of binding without $(NH_4)_2SO_4$] is depicted in Fig. 4. As the concentration of colchicine increased, the difference in the rate of binding with and without $(NH_4)_2SO_4$ disappeared and the ratio approached unity. But contrary to the above-mentioned observation, in the case of colcemid even at high ligand concentration an appreciable level of stimulation by the salt was still maintained. Such an observation focuses the difference in behaviour of colchicine and colcemid towards their binding to tubulin and cannot be explained by the rate effect.

Apparent affinity constants

A Scatchard plot of colcemid binding to tubulin was obtained in the presence of 1.6 M ammonium sulfate to discover whether the enhanced binding was due to a change in the number of binding sites or in the apparent affinity constant. It has already been shown (Fig. 3) that, unlike colchicine, colcemid binds to tubulin at two different sites. Analysis of the colcemid-tubulin interaction on the assumption of a two non-interacting sites model yielded one high-affinity site and one low-affinity site (inset Fig. 5). Surprisingly, the nonlinear Scatchard plot with an upward concavity is converted into a linear plot in the presence of ammonium sulfate without a change in the total number of binding sites (Fig. 5). This linear plot suggested the single class of binding site. Presumably, the original high-affinity site of tubulin for colcemid binding (K_a, 0.7×10^5 M^{-1} at 37 °C) remains unaffected whereas the low-affinity site (K_b, 1.2×10^4 M^{-1}) is converted into the high-affinity one in the presence of ammonium sulfate.

It is known that colcemid and colchicine are mutually competitive inhibitors. Since the competition experiment was carried out at relatively low colcemid concentration [9], it is apparent that the high-affinity site of colcemid competes for the colchicine binding site of tubulin. Thus, the question arises as to whether the low-affinity site of tubulin for colcemid binding recognizes colchicine at all, and, if not, what happens when it is converted into a high-affinity site after ammonium sulfate treatment. For this, tubulin was first saturated with colchicine by incubating it with drug (200 μM). Next, in one set [^{3}H]colchicine (1 μM) and in another set [^{3}H]colcemid (1 μM) were added and again incubated at 37 °C for different times. It is evident from Fig. 6 that [^{3}H]colcemid binding had occurred significantly and the time of equilibration was about 20 min, whereas in the case of the [^{3}H]colchicine-treated tube absolutely no association of radioactivity with tubulin could be detected. These results clearly indicate that colchicine recognizes only the high-affinity site of colcemid and not the low-affinity site. Interestingly, the binding of [^{3}H]colcemid to tubulin saturated with colchicine is also stimulated by ammonium sulfate, suggesting a specific role of anion on the binding of colcemid to the low-affinity site.

Now the question might be asked why the second site cannot accommodate colchicine. This type of discrimination in binding is however, not surprising because yeast tubulin is known to bind only colcemid. Moreover, it is interesting to note that the affinity for colcemid binding at this (low-affinity) site is about 1.2×10^4 M^{-1} which is comparable with the value for affinity constant (0.5×10^4 M^{-1}) obtained for colcemid binding to yeast (*Saccharomyces cerevisiae*) tubulin as reported earlier by Harber et al. [7].

Thus, is appears from these studies that the mechanism of binding reaction of B-ring analogue of colchicine to tubulin will be determined by the substituents present in the B-ring. We

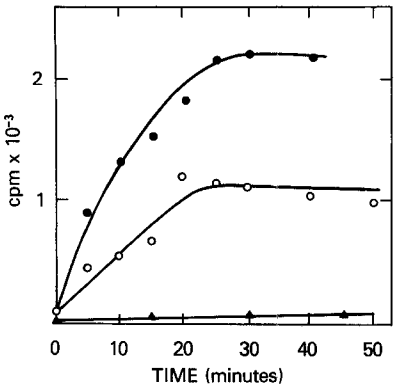

Fig. 6. *Time dependence of binding of [^{3}H]colcemid to a site on tubulin nonsaturable with colchicine.* The reaction mixtures containing tubulin (3.18 μM) and unlabelled colchicine (200 μM) were incubated at 37 °C for 40 min with or without $(NH_4)_2SO_4$ (1.6 M). Next [^{3}H]colchicine or [^{3}H]colcemid (1 μM) was added and further incubated at 37 °C for different periods as indicated. Aliquots were withdrawn at indicated intervals and assayed for bound radioactivity. (○) Bound [^{3}H]colcemid (−salt); (●) bound [^{3}H]colcemid (+salt); (▲) bound [^{3}H]colchicine (±salt)

may conclude, therefore, that tubulin posseses at least two binding sites (domains) one of which is specifically recognized by the B-ring substituents of colchicine and the other one is a high-affinity site which accommodates both colchicine and colcemid and on that site possibly the A and C rings of colchicine interact. Nevertheless by what means the presence of substituents in the B-ring portion of colchicine may alter the binding characteristic of such drugs to tubulin remains to be settled.

We thank Dr Jan Wolff and Dr Danny Sackett of the National Institute of Health (USA) for their comments on this manuscript and Ms Joanne Borthwell for her technical assistance. This work was supported by grants from the Department of Science and Technology, Goverment of India.

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