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BINDING OF COLCHICEINE TO TUBULIN

MECHANISMS OF LIGAND ASSOCIATION WITH TUBULIN

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Abstract—Colchiceine, a closely related structural analog of colchicine possessing a C-ring tropolone, has been shown to be a potent inhibitor of microtubule assembly *in vitro* (I_{50} = 20 μM). The mechanism of inhibition is mediated through binding to tubulin ($K_A = 1.2 \pm 0.7 \times 10^4\ M^{-1}$), although potentially not through the colchicine receptor site. Supporting the hypothesis of an alternate receptor are the observation of colchiceine binding to the isolated colchicine–tubulin complex ($K_A = 2.2 \pm 1.0 \times 10^4\ M^{-1}$), the poor correlation between the competitive inhibition of colchicine binding (K_I = 125 μM) and the inhibition of microtubule assembly, and different structure–activity relationships for colchiceine analogs as compared to the colchicine series.

Tubulin, a 100,000 dalton heterodimer that is the major component of microtubules, is the target for a variety of therapeutic agents. Three distinct, high-affinity drug receptor sites on tubulin have been characterized—the colchicine/podophyllotoxin site, the vinca alkaloid/maytansine site, and the taxol site—and binding to these sites is associated with either substoichiometric inhibition of tubulin assembly or promotion of aberrant tubulin polymerization processes [1, 2]. We have been examining the mechanisms by which ligands bind to the colchicine (**1**) site and the structure–activity relationships for this protein receptor [3, 4]. This site has been shown to accommodate a wide range of structural classes, including podophyllotoxin, benzimidazole and more structurally diverse molecular frameworks, yet often minor modifications of the colchicinoid skeleton have been demonstrated to exhibit dramatic effects on tubulin-binding ability [1, 2, 5, 6]. For example, isocolchicine, a colchicine analog in which the positions of the C ring methoxy and carbonyl are exchanged, is virtually identical to colchicine, yet its affinity for the colchicine binding site is ~500-fold less than colchicine [7] (see Fig. 1 for structures).

Colchiceine (**2**) is a colchicine analog which, by virtue of its tropolone C ring, may exist in two tautomeric forms corresponding to either a colchicine or isocolchicine configuration. Infrared and optical rotation studies, as well as product distribution after alkylation of the tropolone ring, suggest that this analog exists primarily in the iso form [8]. Like isocolchicine, colchiceine inhibits [^{3}H]colchicine binding to tubulin to only a minor extent (2.1 and 6.6% for isocolchicine and colchiceine, respectively, compared to 82.1% for colchicine). Yet its potency in antimitotic and antigout assays is significantly greater than other colchicinoids that weakly bind to the colchicine site [5]. To understand these paradoxical findings we have examined the binding to tubulin of colchiceine and the effect of colchiceine analogs on microtubule assembly *in vitro*. Our data suggest that colchiceine inhibits tubulin polymerization by binding to a new receptor site on the tubulin dimer.

EXPERIMENTAL PROCEDURES

Materials. Pipes,† EGTA, dithioerythritol and GTP (Type II-S) were obtained from the Sigma Chemical Co. (St Louis, MO). Phosphocellulose (Whatman P11, Whatman, Inc., Clifton, NJ) was precycled according to the instructions of the manufacturer. [^{3}H]Colchicine (37.2 mCi/mmol) was purchased from DuPont-New England Nuclear Research Products (Wilmington, DE) and [^{3}H]acetic anhydride (500 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). All experiments were performed in PMG buffer (0.1 M Pipes, 2.0 mM EGTA, 1.0 mM $MgSO_4$, 0.1 mM GTP, 2.0 mM dithioerythritol, pH 6.9 at 23°) unless otherwise noted.

Tubulin purification and protein determination. Tubulin, free of MAPS, was prepared from bovine brain by three cycles of assembly/disassembly, followed by chromatography on phosphocellulose [9], and stored in aliquots in liquid nitrogen. Prior to use, the frozen protein was rapidly thawed, centrifuged at 5000 *g* for 10 min to remove denatured protein and then chromatographed on a Sephadex G-25 column equilibrated with PMG buffer or alternate appropriate buffer. Tubulin concentrations were determined [3, 4] and MAPs were isolated [10] as described previously. Due to the established lability of the colchicine binding site, all experiments were

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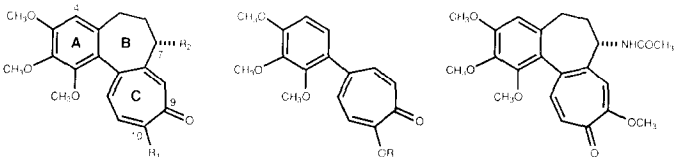
† Abbreviations: EGTA, ethyleneglycol bis-(α-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; MAPs, microtubule associated proteins; Pipes, piperazine-*N,N′*-bis-(2-ethanesulfonic acid); PM buffer, 100 mM Pipes, 1.0 mM $MgSO_4$, 2.0 mM EGTA, pH 6.9; and PMG buffer, 100 mM Pipes, 1.0 mM $MgSO_4$, 2.0 mM EGTA, 0.1 mM GTP, 2.0 mM dithioerythritol, pH 6.9.



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Structure I:

1:	R_1=OCH_3,	R_2=$NHCOCH_3$	: colchicine
2:	R_1=OH,	R_2=$NHCOCH_3$	: colchiceine
3:	R_1=OCH_3,	R_2=H	: deacetamidocolchicine
4:	R_1=OH,	R_2=H	: deacetamidocolchiceine
5:	R_1=OCH_3,	R_2=$NHCH_3$	: demecolcine
6:	R_1=OH,	R_2=$NHCH_3$	: demecolceine
7:	R_1=OCH_3,	R_2=$NHCOCF_3$	: N-deacetyl-N-trifluoroacetylcolchicine
8:	R_1=OH,	R_2=$NHCOCF_3$	: N-deacetyl-N-trifluoroacetylcolchiceine
9:	R_1=OCH_3,	R_2=NH_2	: N-deacetylcolchicine
10:	R_1=OH,	R_2=NH_2	: N-deacetylcolchiceine

Structure II:

11: R=CH_3 : 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone
12: R=H : 5-(2',3',4'-trimethoxyphenyl)-α-tropolone

13: isocolchicine

Fig. 1. Structures of colchicine and analogs.

performed within 6 hr after the protein was initially thawed.

Synthesis of colchicinoids. Colchiceine was prepared via semi-synthesis from colchicine [11], and its structure and purity (>99%) were confirmed by ^1H and ^{13}C NMR and mass and infrared spectroscopic analyses. [^{3}H]Colchiceine (730 μCi/mmol), prepared from trimethylcolchicinic acid [12] by acetylation with [^{3}H]acetic anhydride, possessed >99% radiopurity. Its structure was confirmed by ^1H NMR and mass spectrometry. [^{3}H]Colchiceine and unlabeled colchiceine inhibited microtubule assembly to the same extent (*vide infra*).

The other colchicine derivatives used in this study were prepared using literature procedures [13]. Colchiceine analogs were prepared by mild acid hydrolysis of the corresponding colchicine derivative.

Competitive binding assays. The ability of colchiceine and other colchicine analogs to inhibit [^{3}H]colchicine binding to tubulin was assessed by the following method. Tubulin, the ligand to be tested, and [^{3}H]colchicine were incubated together for 1.5 hr at 37°. Protein-bound ligand was separated from unbound ligand according to the method of Penefsky [14]. In this method, 1.0 mL Sephadex G-50 (fine) columns were prepared and centrifuged for 2 min at 900 *g*. Aliquots of the incubation mixtures (up to 100 μL) were applied to the columns, which were then centrifuged again for 2 min at 900 *g*. The effluent was then analyzed for [^{3}H]colchicine by scintillation spectrometry. Tubulin concentrations as low as 1 μM were successfully used with this technique, although we have found that tubulin concentrations of 5 μM or greater yielded the highest recovery of protein. Control experiments determined the levels of [^{3}H]colchicine in the effluent to be negligible.

Although the procedure for separating the bound ligand differs, we have found that K_I values for allocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone determined using this procedure to be virtually identical to the values reported for these ligands using the filter disk method [5]. Furthermore, essentially no radioactve ligand was found in the column effluent in the absence of tubulin at concentrations up to 200 μM [^{3}H]ligand. We therefore conclude that the filter disk assay and the present method provide equivalent results.

Direct measurement of [^{3}H]colchiceine binding to tubulin. The extent of [^{3}H]colchiceine binding to tubulin was assessed using the gel filtration binding technique described above. In these experiments, various amounts of [^{3}H]colchiceine were incubated with a fixed concentration of tubulin (2.8 μM) at 37° for 45 min. The incubation time was chosen on the basis of an experiment in which the binding of 20 μM [^{3}H]colchiceine to 3 μM tubulin was determined as a function of time. About 60% of the maximum amount of [^{3}H]colchiceine was bound to tubulin after a 1-min incubation time, and saturation was seen after 30 min at 37°. The binding process was quenched by cooling the samples on ice, and 100-μL aliquots were applied to prepared G-50 columns. Aliquots of the effluent were analyzed for tubulin-bound [^{3}H]colchiceine by scintillation spectrometry. Control experiments were performed to determine the levels of [^{3}H]colchiceine in the absence of tubulin (generally negligible) and the concentration of tubulin in the effluent.

The association of [^{3}H]colchiceine with the tubulin–colchicine complex was determined as outlined above, except that a preformed tubulin–colchicine complex was substituted for tubulin. The colchicine–tubulin complex was prepared by incubating tubulin (96 μM) with colchicine (810 μM) for 30 min at 37°. Unbound colchicine was removed by gel filtration prior to addition of [^{3}H]colchiceine.

The association parameters for colchiceine–tubulin and colchiceine–tubulin/colchicine complex binding were determined by nonlinear regression analysis using the program LIGAND [15].

Table 1. Association with tubulin and inhibition of microtubule assembly by colchicine analogs

Compound	K_A ($\times 10^5$), M^{-1}, 23°	K_I,* μM	I_{50},† μM
Colchicine (**1**)	30 [6]–2.0 [4]	2.5	2
Colchiceine (**2**)	0.1	125	20
2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (**11**) [4]	3.5	10	4
Deacetamidocolchicine (**3**)	~16 [16]	5 [17]	3
Isocolchicine (**13**) [7]	.06	400	1100
α-Tropolone [18]	~.01	ND‡	>200
N-Acetylmescaline [18]	~.004	ND‡	>2500

* Inhibition constant.

† Value for 50% inhibition of assembly of 2.0 mg/mL purified tubulin except in the case of *N*-acetylmescaline, where microtubule protein was used.

‡ Not determined.

Effect of colchiceine on reversibility of the colchicine–tubulin complex. The ability of colchiceine to displace [^{3}H]colchicine bound to tubulin was determined as follows. The colchicine–tubulin complex was formed by incubating tubulin (7 μM) and [^{3}H]colchicine (7 μM) at 37° for 2 hr. Samples were prepared containing the [^{3}H]colchicine–tubulin complex (5 μM) and various concentrations (up to 100 μM) of unlabeled colchiceine or colchicine and incubated for 1 hr at 37°. The binding process was quenched by cooling the samples on ice, and the [^{3}H]colchicine–tubulin complex was separated from unbound ligand by the rapid gel filtration method described above. Aliquots of the effluent were analyzed for tubulin-bound [^{3}H]colchicine by scintillation spectrometry. The amount of [^{3}H]colchicine incorporated in the presence of the unlabeled colchiceine or colchicine was compared to the amount incorporated in the control in which no unlabeled ligand was added.

Assays for the inhibition of microtubule assembly. Tubulin polymerization was performed in PM buffer (0.1 M Pipes, 2.0 mM EGTA, 1.0 mM $MgSO_4$, pH 6.9) using a tubulin concentration of 2.0 ± 0.1 mg/mL. The tubulin and tubulin/colchicinoid solutions were preincubated at room temperature for 20 min and then cooled to 4° in a cuvette placed in a thermostatted cell holder. GTP was added to a concentration of 1.0 mM and either MAPs [20% (w/w) of tubulin] or glycerol (to a final concentration of 3.2 M) was added. Assembly was initiated by rapidly raising the temperature to 37° and the progress of tubulin polymerization was monitored for 30 min by the increase in turbidity of the solution as observed by increased optical density at 400 nm.

RESULTS

Data for the inhibition of microtubule assembly (I_{50}), the inhibition of [^{3}H]colchicine binding to tubulin (K_I) and the association constant of the ligand–tubulin complex (K_A) are compiled in Table 1 for colchiceine (**2**), colchicine (**1**) and several analogs. Colchiceine inhibited the polymerization of tubulin into microtubules at 50% of assembly controls (I_{50})

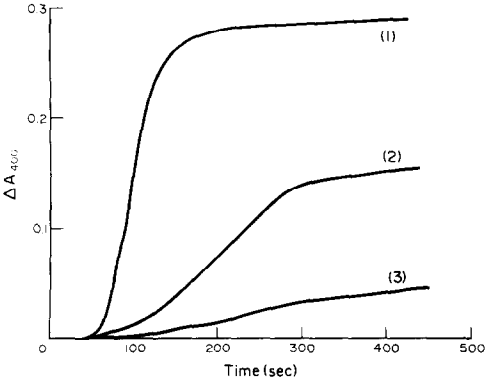

Fig. 2. Inhibition of microtubule polymerization *in vitro* by colchiceine. Microtubule assembly was initiated by addition of MAPs as described in Experimental Procedures. Key: (1) no colchiceine; (2) 20 μM colchiceine; and (3) 60 μM colchiceine.

at a concentration of 20 μM (Fig. 2), which is 10-fold greater than that required by colchicine. The inhibition of microtubule assembly was shown to be a consequence of association with *tubulin*, rather than MAPs, through an alternate series of I_{50} determinations employing glycerol to initiate polymerization, which provided analogous results. As tropolone derivatives possess established metal ion chelation abilities (as contrasted with their methoxytropone counter parts, e.g. **2** vs **1**) [19, 20], the possibility of a specific metal ion coordination effect as causal in the inhibition of microtubule assembly was investigated. Tropolone, a single ring analog of colchiceine which retains the magnesium chelating ability of the parent molecule, is at least 10-fold less active in inhibiting microtubule polymerization than colchiceine (Table 1). Furthermore, the bicyclic analog of colchiceine (compound **11** in Table 2) displayed little inhibitory activity. These data indicate that metal ion coordination is not singly responsible for the inhibition of microtubule polymerization by colchiceine.

Colchiceine was a weak competitive inhibitor of colchicine binding to tubulin, as illustrated by the K_I

Table 2. Structure–activity relationships of colchicine and colchiceine derivatives on microtubule assembly *in vitro*

Colchicine derivatives Compound*	I_{50},† μM	Colchiceine derivatives Compound*	I_{50},† μM
1	2	**2**	20
3	3	**4**	>100‡
5	7	**6**	76
7	1.5	**8**	14
9	6	**10**	150
11	4	**12**	>100‡

* See Fig. 1 for structures.

† Concentration at which the control value of MAP-induced microtubule assembly was inhibited by 50% (see Experimental Procedures).

‡ Not soluble at higher concentrations.

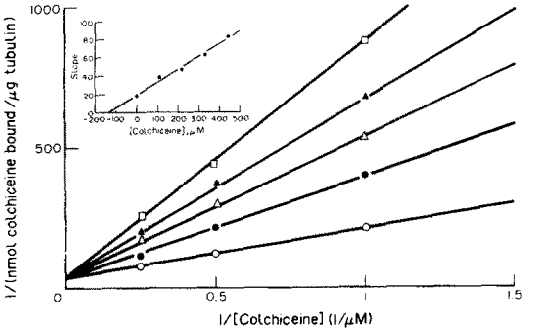

Fig. 3. Lineweaver–Burk plot demonstrating the effect of colchiceine on [^{3}H]colchicine binding to tubulin. The reciprocal of bound [^{3}H]colchicine is plotted against the reciprocal of the total concentration of [^{3}H]colchicine at several concentrations of colchiceine: (○—○, 0 μM); (●—●, 110 μM); (△—△, 220 μM); (▲—▲, 330 μM); and (□—□, 440 μM). Inset: Slopes of the lines of the Lineweaver–Burk plot versus total concentration of colchiceine.

value of 125 μM (Fig. 3), which is 50-fold greater than the value for colchicine. The association of colchiceine with tubulin was directly assessed by examining the binding of [^{3}H]colchiceine to tubulin (Fig. 4). At low concentrations of colchiceine (<~100 μM), the association constant for the colchiceine–tubulin complex was determined to be $1.2 \pm 0.7 \times 10^4\ M^{-1}$ and a stoichiometry of 2.5 ± 1.2 binding sites/tubulin dimer was found. We were unable to fit the data to models in which colchiceine binding sites are of different classes; thus, the association constant may represent an average of the association constants for the different sites. At higher concentrations of colchiceine, additional binding sites were apparent, but determination of K_A values for these sites was not possible with accuracy.

The stoichiometry for the colchiceine–tubulin association indicates that colchiceine may interact with tubulin at sites other than the colchicine receptor site. Therefore, the binding of colchiceine to the tubulin–colchicine complex was examined (Fig.5). As the Scatchard plot appeared to curve, we attempted to analyze the data in terms of two classes of binding sites. Even if the low concentration points were omitted, we were unable to obtain a fit for the data for greater than one class of binding sites. Analysis of all the points yielded an association constant of $2.1 \pm 1.0 \times 10^4\ M^{-1}$ and a stoichiometry of 0.86 ± 0.25 sites/tubulin dimer.

To assure that colchiceine binding to the tubulin–colchicine complex was not the result of colchiceine displacing tubulin-bound colchicine, the effect of colchiceine on the reversibility of the tubulin–colchicine complex was examined. Both colchicine and colchiceine at concentrations up to 100 μM displaced less than 5% of tubulin-bound [^{3}H]colchicine, indicating that colchiceine does not affect the reversibility of the tubulin–colchicine complex.

To assess whether the effects of colchiceine on microtubule assembly are mediated through the colchicine site, the efficacies of several colchicine and colchiceine derivatives on inhibition of microtubule polymerization were studied (Table 2). It is seen that the structure–activity relationship of the colchiceine derivatives is quite different from that of the colchicine series.

DISCUSSION

Studies of the pharmacology and medicinal chemistry of colchiceine and C-ring tropolonic colchicine analogs have promoted the general belief that the colchiceine class does not effectively bind to tubulin or inhibit microtubule polymerization; colchiceine is "inactive" in assays for competitive binding with [^{3}H]colchicine to tubulin and has a relatively low toxicity for rodents [5]. However, colchiceine has been reported to exhibit a number of biological effects characteristic of microtubule disruption, such as suppression of sodium urate-induced edema [21], the equipotent (with colchicine) inhibition of fast axonal transport in isolated sciatic nerve and the inhibition of microtubule protein assembly at concentrations 5- to 10-fold higher than colchicine [22]. The data presented here demonstrate that colchiceine *is* a potent inhibitor of tubulin assembly into microtubules and that the mechanism of inhibition is mediated through binding to tubulin, not complexation with MAPs or metal ions. Several observations suggest that colchiceine-mediated inhibition of microtubule assembly may not be solely a consequence of association at the colchicine binding site.

The central finding is the unequivocal identification of colchiceine interaction at a binding site distinct from the high-affinity colchicine receptor. Colchiceine binds with moderate affinity to at least two sites on tubulin, one of which is apparently blocked by colchicine. Evidence which supports an alternate site as being responsible for the effectiveness of colchiceine is first noted by examining the relationship between inhibition of tubulin polymerization and binding to the colchicine site (Table 1). In general, a good correlation was seen between affinity for the colchicine site on tubulin and assembly inhibition. Colchiceine, however, showed strong inhibition of tubulin polymerization while only weakly inhibiting [^{3}H]colchicine binding. Furthermore, structural modifications of the colchiceine skeleton affected its potency in a manner different

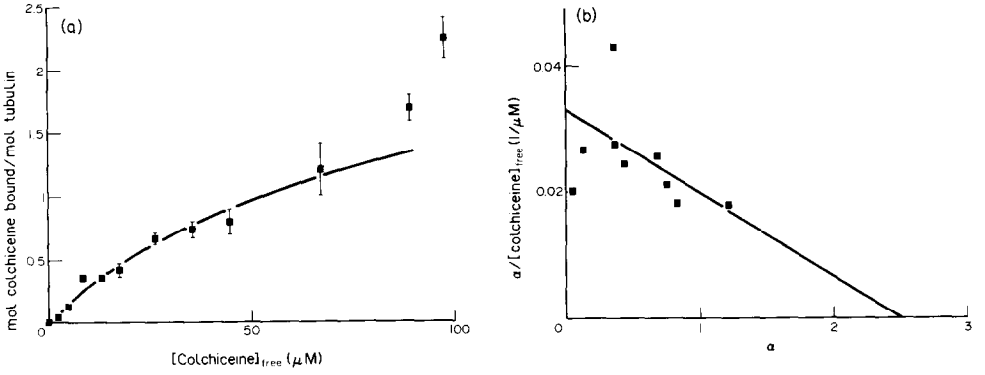

Fig. 4. Binding of [^{3}H]colchiceine to tubulin. Tubulin (2.8 μM) was incubated for 45 min at 37° with increasing amounts of [^{3}H]colchiceine. (a) The moles of [^{3}H]colchiceine bound per mole tubulin is plotted against the concentration of free [^{3}H]colchiceine, determined by subtraction of bound from total concentration of [^{3}H]colchiceine. (b) Scatchard plot of the data in panel a. The number of moles of [^{3}H]colchiceine bound per mole of tubulin is represented by the symbol α. The solid line in the plot was calculated by nonlinear regression analysis using the program LIGAND [15]. The parameters returned by the program were also employed to calculate the solid line for the binding curve in panel a.

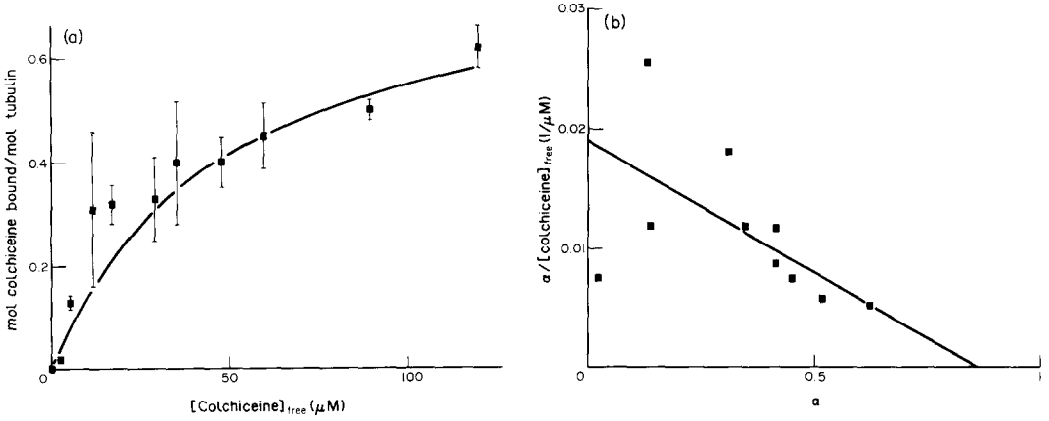

Fig. 5. Binding of [^{3}H]colchiceine to the colchicine–tubulin complex. Tubulin (2.9 μM) which had been saturated previously with colchicine, as described in Experimental Procedures, was incubated with increasing amounts of [^{3}H]colchiceine for 45 min at 37°. (a) The moles of [^{3}H]colchiceine bound per mole tubulin is plotted against the concentration of free [^{3}H]colchiceine, determined by subtraction of bound from total concentration of [^{3}H]colchiceine. (b) Scatchard plot of the data in panel a. The number of moles of [^{3}H]colchiceine bound per mole of tubulin is represented by the symbol α. The solid line in the plot was calculated by nonlinear regression analysis using the program LIGAND [15]. The parameters returned by the program were also employed to calculate the solid line for the binding curve in panel a.

from similar structural changes in colchicine (Table 2). In particular, the data indicate than an intact B ring and a substituent at the C-7 position are required for activity in the colchiceine series. These observations are most dramatic for the bicyclic analogs of colchicine (**11**) and colchiceine (**12**). Compound **11** was nearly as active as colchicine despite removal of the B ring of the parent molecule. The tropolone derivative **12**, however, was essentially inactive.

The suggestion of alternate binding sites on tubulin for colchicinoids is not unique. Ray *et al.* [23] observed two binding sites on tubulin for colcemid—a high-affinity site ($K_A = \sim 7.0 \times 10^4\ M^{-1}$), which competitively bound colchicine, and a low-affinity site ($K_A = \sim 1.2 \times 10^4\ M^{-1}$). Ringel and Sternlicht [12] observed a high-affinity site ($K_A = 2.0 \times 10^5\ M^{-1}$) and a low-affinity site(s) ($K_A = \sim 1.6 \times 10^3\ M^{-1}$) for colchicine association with tubulin and ascribed significance to the(se) low-affinity binding site(s) with regard to the influence of singular or multiple occupancy of the site(s) on the mechanisms operating in the inhibition of tubulin polymerization. They postulated that colchicine concentrations sufficient to bind small levels of the low-affiity site(s) precipitate an abrupt inhibition of tubulin polymerization, in contrast to the mechanism of inhibition that proceeds with exclusive colchicine site occupancy. Additionally, Deinum and Lincoln [24] have found that an allocolchicine spin label, a colchicinoid with an aromatic C ring, binds to an

additional site(s) on the tubulin dimer as well as associating with the colchicine binding site.

It is possible that the alternate (low-affinity) colchicinoid binding sites identified during investigations of the binding to tubulin of colcemid, colchicine and allocolchicine may prove to be the receptor site for colchiceine identified in these investigations. Our preliminary studies suggest that this site is not the Vinca alkaloid receptor site. At concentrations of 3.0 μM tubulin and 50 μM [^{3}H]colchiceine, concentrations of vinblastine up to 210 μM had no effect on the amount of colchiceine bound to tubulin (data not shown). The data presented here demonstrate that colchiceine is a potent inhibitor of microtubule assembly, and this inhibition may be mediated through a distinct receptor with unexplored potential for medicinal chemical development.

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