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Tubulin Binding Affinities of Podophyllotoxin and Colchicine Analogues

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SUMMARY

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The lignan podophyllotoxin competitively inhibits colchicine binding to tubulin. The ability of 12 podophyllotoxin and three colchicine analogues to inhibit colchicine binding to mouse brain tubulin was investigated in order to identify drugs with high affinity for the colchicine binding site on tubulin. Colchicine binding was assayed by the DEAE-cellulose filter paper method. Results indicated that podophyllotoxin binds to tubulin more rapidly and in less temperature-dependent fashion than colchicine. All active drug analogues were competitive inhibitors. β-Peltatin was found to have a significantly greater affinity for mouse brain tubulin than either podophyllotoxin or colchicine. Analogues containing hydrophilic substitutions had greatly reduced tubulin binding activity, as did stereoisomers of podophyllotoxin. Other results suggest that the conformation about the lactone ring on podophyllotoxin may be of importance in determining tubulin binding activity. These results are consistent with the hypothesis that the colchicine binding site is located in a hydrophobic pocket. Tubulin binding assays *in vitro* are suggested as useful steps in the screening of antitumor agents.

INTRODUCTION

The alkaloid colchicine and the lignan podophyllotoxin produce metaphase arrest in dividing cells (see refs. 1 and 2 for reviews). Colchicine has been shown to bind to microtubule protein, tubulin, which is a major component of the mitotic apparatus (3). Colchicine binding to tubulin results in positive enthalpy and entropy changes and a fairly large free energy change, indicating that the binding site may be located in a hydrophobic or nonpolar pocket (4). Podophyllotoxin, a bioactive plant lignan isolated from the May apple, *Podophyllum peltatum*, behaves as a competitive inhibitor of colchicine binding to tubulin (5, 6). In addition to their effect on mitosis, colchicine and podophyllotoxin inhibit a variety of other microtubule-dependent processes, including fast axoplasmic transport (7), long saltatory intracellular movements (8), and cilia regeneration (9). Thus it is believed that these drugs bind to tubulin at a site which is critical for the polymerization of microtubules.

Colchicine has been studied more extensively than podophyllotoxin; yet there is evidence that podophyllotoxin has a greater affinity than colchicine for several vertebrate tubulins (10-12). Although col-

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chicine and podophyllotoxin are competitive inhibitors, the structures of these two molecules differ sufficiently to suggest that some degree of structural variation may be accommodated at their binding site. This study was undertaken to investigate the tubulin binding properties of podophyllotoxin and colchicine analogues in order to gain insight into the molecular requirements for drug binding at this important site on the tubulin molecule. Mouse brain tubulin was chosen because podophyllotoxin analogues have been extensively analyzed in mouse tumors *in vitro* (13) and *in vivo* (14). Evaluation of drug affinity at this site may be useful in locating improved agents for chemotherapy. Clinically, tubulin binding drugs have been suggested as useful in the treatment of gout (15) and cancer (16, 17).

METHODS AND MATERIALS

Preparation of mouse brain tubulin. Freshly dissected mouse brains were homogenized on ice, 1 part by weight to 5 parts by volume of 0.01 M phosphate buffer, pH 6.8, containing 0.01 M GTP and 10% glycerol. The homogenate was centrifuged at 40,000 × *g* for 1 hr. The supernatant, defined as crude mouse brain extract, contained 2–4 mg of protein per milliliter as assayed by the method of Lowry *et al.* (18). This protein contained 6–13% tubulin, defined as colchicine-binding material of 120,000 mol wt.

Colchicine binding assay. The DEAE-cellulose filter paper assay of Borisy (19) was used to determine the colchicine binding activity of crude brain extract. Incubation mixtures (0.25 ml) consisted of 0.05 ml of crude brain extract, [^{3}H]colchicine, and other drugs where indicated. Drugs with low water solubility were added to the incubation mixtures in ethanol or acetone at a final concentration of less than 1%, a level of solvent which had no effect on the colchicine binding assay. These mixtures were incubated for 3 hr at 37° except where otherwise noted. Bound and free colchicine were then separated by adsorption of tubulin to DEAE-cellulose filters. Filters were washed four times with 10-ml portions of 0.01 M phosphate buffer, pH 6.8, containing 0.01 M NaCl. Damp filters were transferred to scintillation vials containing 10 ml of scintillation fluid consisting of Triton X-100 (1 part), xylene (2 parts), 2,5-diphenyloxazole (3 g/liter), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.2 g/liter) (20). After 12 hr had been allowed for desorption of [^{3}H]colchicine, the filters were counted in a Packard Tri-Carb model 3200 scintillation counter. Counting efficiency, determined by the addition of a ^3H_2O internal standard, was 22%.

K_i values for the inhibition of colchicine binding to tubulin by the inhibitors used in this study were computed from least-square regression lines of Linweaver-Burk plots for the binding of colchicine to tubulin. The RASS statistical package run on an IBM 370 computer at the Boston University Computer Center was used for these computations.

Electron microscopy. Crude mouse brain extract was prepared in polymerization buffer as described by Borisy and Olmsted (21). After 30 min of incubation at 37°, samples were placed on 400-mesh, Formvar-coated grids and stained with 1% uranyl acetate. Grids were examined under a JEM 100B electron microscope for the presence of microtubules.

DEAE-Sephadex chromatography. Samples of 0.05 ml of crude mouse brain extract were incubated with [^{3}H]colchicine (18.4 Ci/mmole) for 2–3 hr and applied to 1 × 4 cm DEAE-Sephadex columns equilibrated with 0.10 M NaCl in phosphate buffer, pH 6.8. Colchicine-bound material was eluted by washing the column with buffer solutions of increasing ionic strength.

Materials. [^{3}H]Colchicine (18.4 Ci/mmole) was purchased from New England Nuclear and diluted to specific activities as low as 0.02 Ci/mmole with unlabeled colchicine purchased from Sigma Chemical Company. Podophyllotoxin was obtained from Aldrich Chemical Company. Analogues of colchicine and podophyllotoxin were generous gifts from the following individuals: Dr. A. von Wartburg, Sandoz, Ltd., Basel, β-peltatin, picropodophyllin, 4′-demethylpodophyllotoxin, desoxypodophyllotoxin, epipodophyllotoxin, β-peltatin β-D-glucopyranoside, and podophyllotoxin β-D-glucopyranoside; Dr. Walter

Gensler, Boston University, anhydropodophyllol; Dr. Jack Cole, University of Arizona, β-peltatin *A*-methyl ether; Dr. T. N. Margulis, University of Massachusetts, Boston, *N*-desacetylthiocolchicine and thiocolchicoside; Dr. Harry B. Wood, National Cancer Institute, podophyllic acid and podophyllic acid 2-ethylhydrazide. Podophyllic acid (NSC 35475) is most likely in the picro conformation (22) and is referred to here as picropodophyllic acid. Trifluralin was a gift of Dr. D. Hess, University of California, Davis, and potassium plicaticate was donated by Dr. J. A. F. Gardner, University of British Colombia, Vancouver. 1,2,3-Trimethoxybenzene and corydaline were purchased from Aldrich, and melatonin, reserpine, GTP, and 3,4,5-trimethoxybenzoic acid, from Sigma.

RESULTS

Properties of mouse brain tubulin. To verify that the 40,000 × *g* supernatant of homogenized mouse brain did contain a colchicine-binding fraction with properties expected of tubulin, this material was examined to determine colchicine binding activity, elution of colchicine-bound material from DEAE-Sephadex, and ability to form microtubules *in vitro*. Incubation of [^{3}H]colchicine with crude mouse brain extract at 37° for 3.5 hr produced colchicine-binding material (K_m = 1.1 μM) determined by the DEAE-cellulose filter paper assay. Podophyllotoxin competitively inhibited this interaction (K_i = 0.51 μM). Samples of crude mouse brain extract were incubated with [^{3}H]colchicine and applied to a DEAE-Sephadex column as described in METHODS AND MATERIALS. The colchicine-bound material was eluted at 0.5–0.6 M NaCl. The polymerization of mouse brain microtubules *in vitro* was observed. After incubation in a buffer solution containing GTP and glycerol and treatment as described in METHODS AND MATERIALS, 260-A (±10 A) microtubules were clearly visible under the electron microscope at 10,000 × magnification. These results indicate that the colchicine-binding moiety from mouse brain is similar to that isolated from other vertebrate sources.

Differences between colchicine and podophyllotoxin binding to tubulin. Basic differences are apparent in the manner in which these two drugs bind mouse brain tubulin. At less than saturating concentrations of colchicine (significantly less than the K_m), the rate of colchicine binding to tubulin is relatively slow. At 37° more than 3 hr of incubation are required for maximum binding. In contrast, podophyllotoxin binds rapidly to this site on tubulin. (The percentage inhibition of colchicine binding to tubulin in the presence of podophyllotoxin does not change significantly over the 3-hr period for maximum colchicine binding.) The half-time for podophyllotoxin binding to tubulin at this site is estimated to be less than 0.5 hr.

The binding of colchicine to mouse brain tubulin is essentially irreversible over the lifetime of the tubulin preparation *in vitro*. Three hours after 50-fold dilution of the colchicine-tubulin incubation mixtures, over 80% of the colchicine remained bound to tubulin. Podophyllotoxin, however, dissociates from mouse brain tubulin quite rapidly (Fig. 1). To measure this rate, tubulin was incubated with podophyllotoxin for 2 hr, followed by dilution of the protein in the presence of [^{3}H]colchicine and additional podophyllotoxin where indicated. The resulting pattern of colchicine binding in 10-fold diluted samples (Curve *B*) rapidly approached that found for samples maintained at the lower podophyllotoxin concentration (Curve *A*). The half-time for dissociation of podophyllotoxin from tubulin was judged to be less than 30 min.

In addition, the binding of colchicine to mouse brain tubulin is more sensitive to temperature variation than podophyllotoxin binding. Whereas colchicine binding to tubulin was reduced approximately 3-fold at 24°, compared to the 37° value, podophyllotoxin inhibition of colchicine binding was only slightly affected by this temperature change. These results demonstrate that podophyllotoxin binding to the colchicine site on tubulin is not influenced by temperature as strongly as colchicine binding.

Inhibition of colchicine binding to tubulin by drug analogues. Twelve analogues

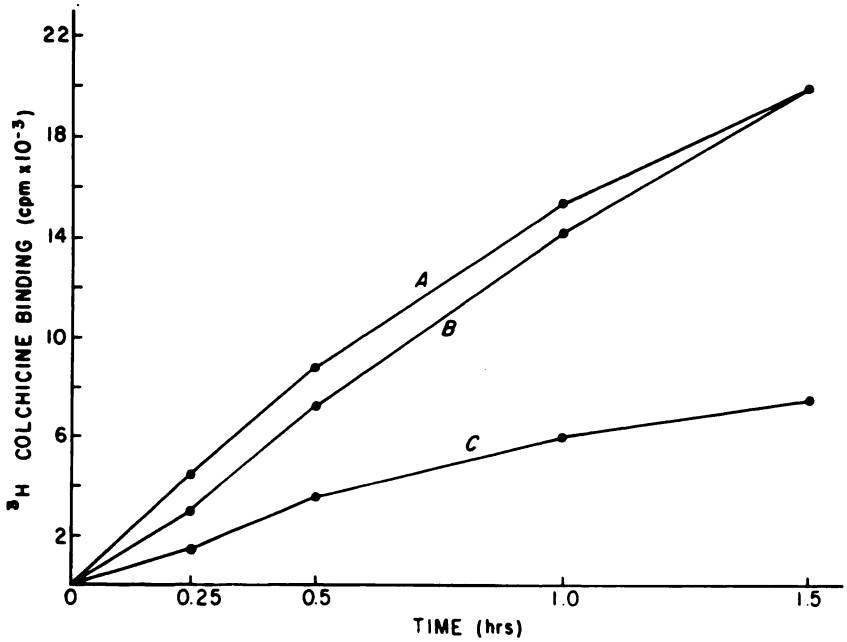


FIG. 1. *Dissociation of podophyllotoxin from mouse brain tubulin*

Crude mouse brain extracts were first incubated with podophyllotoxin for 2 hr. At zero time the protein was diluted 10-fold, and [^{3}H]colchicine, 56 nM (18.4 Ci/mmole), was added. The subsequent colchicine binding rate was measured to determine the effect of the preliminary incubation with podophyllotoxin. Samples were treated as follows: curve *A*, maintained at 80 nM podophyllotoxin throughout the experiment; *B*, first incubated with 800 nM podophyllotoxin, which was diluted to 80 nM at zero time; *C*, maintained at 800 nM podophyllotoxin throughout. Values represent averages of at least two determinations, which did not differ by more than 10%.

of podophyllotoxin and three of colchicine were examined for inhibition of [^{3}H]colchicine binding to mouse brain tubulin by the DEAE-cellulose filter paper assay method. All active analogues were competitive inhibitors. Podophyllotoxin and colchicine analogues were considered inactive if no inhibition of colchicine binding occurred at 1 mM. These studies identified one compound, β-peltatin, as having a demonstrably greater affinity for the colchicine binding site on tubulin than either colchicine or podophyllotoxin. Figure 2 is a double-reciprocal plot of the inhibition produced by 2 μM podophyllotoxin and β-peltatin. This assay method indicates that β-peltatin has a greater affinity for this site on mouse brain tubulin than any other compound tested to date. Table 1 compares the inhibition constants calculated for podophyllotoxin, β-peltatin, and other analogues containing minor substitutions on the A, B, and C rings. The inhibition constants (K_i) listed there should approximate the dissociation constants for these compounds with tubulin. Most of the substitutions in Table 1 did not produce large alterations in tubulin binding activity. The similarity in the K_i values of podophyllotoxin and desoxypodophyllotoxin suggest that the increased tubulin binding activity of β-peltatin is more related to the presence of a hydroxyl group on ring A than to the lack of one on ring B. 4'-Demethoxypodophyllotoxin is also nearly equal to podophyllotoxin in tubulin binding ability. This result is of interest because both colchicine and podophyllotoxin contain 3,4,5-trimethoxybenzyl groups. Apparently the 4'-methoxy group is not required for activity. The stereoisomer, epipodophyllotoxin, displays notably reduced binding activity. That the stereochemistry of the ligand is important for its interaction with tubulin is further illustrated by the low tubulin binding activity of picropodo-

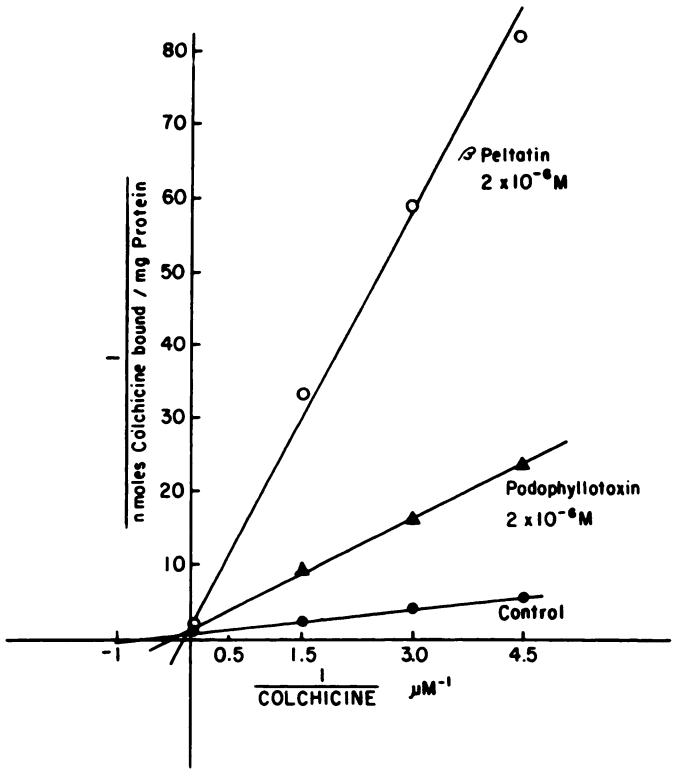


FIG. 2. *Competitive inhibition of colchicine binding to mouse brain tubulin by β-peltatin and podophyllotoxin*

The double-reciprocal plot of colchicine binding to mouse brain tubulin was constructed as described in METHODS AND MATERIALS.

phyllin (Table 2). In addition, the relatively high K_i values of all the analogues in Table 2 are evidence that the lactone ring is important for this interaction. A striking decrease in tubulin binding activity is also observed with the glucopyranoside analogues of podophyllotoxin listed in Table 3. All these compounds are less than 1% as active as podophyllotoxin. Either the large size or hydrophilic character of the substitutions may reduce the affinity for tubulin. The low activity of the sugar-substituted compounds is also evident with a colchicine analogue, thiocolchicoside (Table 4). Of the other colchicine analogues, only *N*-desacetylthiocolchicine is an effective inhibitor of colchicine binding.

Several additional compounds not related to colchicine or podophyllotoxin were assayed for the inhibition of colchicine binding to tubulin by the DEAE-cellulose filter paper assay. The following compounds, listed with the highest concentrations tested, were not active inhibitors: 1,2,3-trimethoxybenzene (0.50 mM), 3,4,5-trimethoxybenzoic acid (0.30 mM), corydaline (0.05 mM), reserpine (0.05 mM), potassium plicaticate (1.0 mM), trifluralin (0.20 mM), and melatonin (2.0 mM). Since melatonin and trifluralin are known to be mitotic inhibitors, the results shown here suggest that the antimitotic properties of these two compounds do not involve a strong interaction with tubulin at the colchicine binding site.

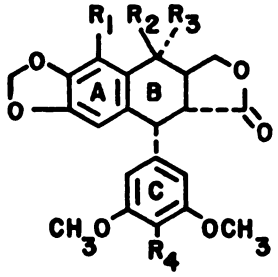
DISCUSSION

Examination of the properties of mouse brain tubulin indicated several similarities with tubulin from other sources. The competitive inhibition by podophyllotoxin for the colchicine site on tubulin and the greater affinity of podophyllotoxin for this site have been observed with chick (10),

TABLE 1

Tubulin binding affinities of podophyllotoxin analogues with minor substitutions in rings A, B, and C

K_i values were determined from Linweaver-Burk plots of [^{3}H]colchicine binding to mouse brain tubulin as described in METHODS AND MATERIALS. Values are averages of three determinations, which did not differ by more than 10%.



Analog	R_1	R_2	R_3	R_4	K_i (μM)
podophyllotoxin	H	H	OH	OCH_3	0.51
β-peltatin	OH	H	H	OCH_3	0.12
β-peltatin-A-methyl ether	OCH_3	H	H	OCH_3	0.57
desoxypodophyllotoxin	H	H	H	OCH_3	0.54
epipodophyllotoxin	H	OH	H	OCH_3	1.7
4'demethylpodophyllotoxin	H	H	OH	OH	0.65

rat (11), and pig (12) brain material. However, Wilson (23) has found that human brain tubulin has a greater affinity for colchicine than podophyllotoxin. These studies reported colchicine dissociation constants between 0.34 and 2.0 μM, and podophyllotoxin dissociation constants between 0.28 and 0.83 μM. Similarities in tubulin-drug association among proteins isolated from different sources is not surprising in view of the conservative amino acid sequence of this protein (24).

Mouse brain tubulin is eluted from DEAE-cellulose at approximately the same ionic strength reported for tubulin from yeast (25) and chick brain (26). Although tubulin was the major component eluted from the ion-exchange column at 0.5–0.6 M NaCl, this method was not found useful in the purification of mouse brain tubulin because the chloride ionic strength required to elute the protein from the column produced a rapid decay of colchicine binding activity. A similar effect has been observed with other tubulins (27).

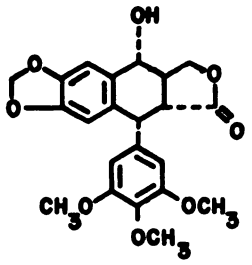
Another tubulin purification procedure, using repeated cycles of polymerization (28), was also unsuccessful. The appearance of microtubules prepared from mouse brain tubulin under the electron microscope is an indication that functionally active tubulin protein was isolated. However, when repeated cycles of polymerization and depolymerization were attempted, less than 10% of the colchicine binding activity was recovered after two cycles. In our hands mouse brain microtubules were difficult to depolymerize even when maintained at 0°.

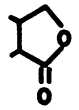
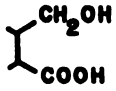
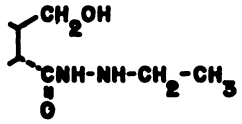
The results of this study and others (23) indicate that colchicine and podophyllotoxin differ significantly in both kinetics and temperature sensitivity of binding vertebrate tubulin. Recently it has been demonstrated that tropolone inhibits colchicine but not podophyllotoxin binding to

TABLE 2

Tubulin binding affinities of podophyllotoxin analogues with substitutions on lactone ring

For details, see the legend to Table 1.



Analog	lactone ring becomes	K_i (μM)
anhydropodophyllol		5.2
picropodophyllin		10
picropodophyllic acid		Inactive
podophyllic acid-2-ethylhydrazide		4.5

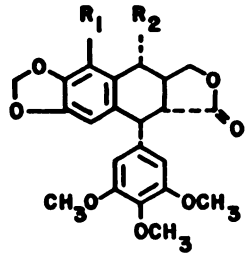
tubulin (29). These observations are evidence that the drugs occupy overlapping rather than identical sites and that hydrophobic forces play a more significant role in colchicine binding. If these differences determined *in vitro* reflect the properties of tubulin *in vivo*, the two drugs may be suitable for different experimental purposes. Podophyllotoxin would be the microtubule inhibitor of choice for a rapid and reversible equilibrium between free and drug-bound tubulin, while colchicine would be more useful in obtaining tight, irreversible binding. At present it is not clear to what extent intracellular microtubule systems respond as these properties predict. Colchicine inhibition of microtubule-dependent processes has been shown to be quickly reversible in several systems, including mitosis (30) and flagellar regeneration (31). However, in at least one case, cell shape development in *Ochromonas dancia* (32), the recovery from colchicine has been shown to be sensitive to cycloheximide, suggesting that microtubule protein cannot reassemble after colchicine treatment. Further investigations of drug activity *in vitro* and *in vivo* are needed to elucidate the exact nature of this effect.

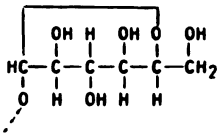
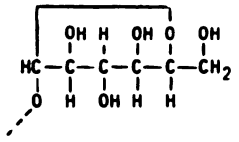
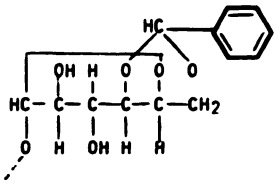
Another important discrepancy between results *in vitro* and *in vivo* with microtubule inhibitors is the effective concentration of these drugs in living systems. Several studies have reported that the concentration of colchicine active *in vivo* is significantly below the K_m determined *in vitro*

TABLE 3

Tubulin binding affinities of podophyllotoxin analogues containing glucopyranoside substitutions

For details, see the legend to Table 1.



Analog	R_1	R_2	K_i (μM)
podophyllotoxin-β-D-glucopyranoside	H		180
β-peltatin-β-D-glucopyranoside		H	310
podophyllotoxin-4,6-O-benzylidene-β-D-glucopyranoside	H		39

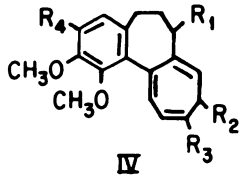
(23, 33). Results with mouse tubulin are in agreement. Stähelin (13) has reported the drug concentrations producing 50% inhibition of mouse tumor cell growth in culture. The values for both colchicine and podophyllotoxin (4 and 5 μg/liter, respectively) are approximately 1% of the dissociation constants reported here. Such data suggest that tubulin-binding drugs disrupt mitosis by preferential binding to a small fraction of the tubulin molecules which are critical for polymerization.

Insight into the nature of the colchicine binding site may be gained from analysis of the relative binding of drug analogues to tubulin. The stereoisomers, epipodophyllotoxin and picropodophyllin, are less active than podophyllotoxin, suggesting physical constraints on the fit of the molecule at the receptor site. The low activity of picropodophyllin has been observed in several systems (5, 7, 12). In fact, since all podophyllotoxin analogues containing substitutions on the lactone ring (Table 2) have reduced affinity for tubulin, this area may be involved in the interaction with tubulin. It has been suggested (34) that the lower oxygen on the lactone ring of podophyllotoxin may be structurally analogous to the tropolone methoxy group on colchicine (R_3 in Table 4). The finding that colchicine analogues without OCH_3 or SCH_3 in this position, such as isocolchicine (11) and demethylisothiocolchicine (Table 4), are inactive as inhibitors of colchicine binding is supportive of this view. Another pattern evident from these studies is that colchicine and podophyllotoxin analogues with hydrophobic substitutions are not high in tubulin binding activity (Tables 2–4).

TABLE 4

Tubulin binding affinities of colchicine analogues

K_m values were determined instead of K_i. Other details were the same as described in Table 1.



IV

COLCHICINE AND ITS ANALOGUES

	R_1	R_2	R_3	R_4	K_i (μM)
Colchicine	$NHC(=O)-CH_3$	=O	OCH_3	OCH_3	1.1[a]
N-desacetyl thiocolchicine	NH_3^+	=O	SCH_3	OCH_3	7.2
Demethylisothiocolchicine	$NHC(=O)-CH_3$	SCH_3	=O	OH	inactive
Thiocolchicoside	$NHC(=O)-CH_3$	=O	SCH_3	$C_6H_{11}O_7$	inactive

Other water-soluble compounds, colchicoside (11) and succinylpodophyllotoxin (12), do not inhibit colchicine binding to brain tubulin. These results are consistent with the idea that the receptor is located in a hydrophobic pocket, as suggested by Bryan (4).

Tubulin binding assays *in vitro* may be useful in the identification of drugs for clinical study. The K_i values reported here have been compared with the effectiveness of these drugs in inhibiting tumor growth in two mouse tumor systems (see ref. 35 for details). Spearman rank order coefficients comparing the inhibition of mastocytoma growth as studied by Stähelin (13) with tubulin binding K_i values indicated that the correlation was significant at $p \leqq 0.01$. When the K_i values were compared with the minimum dose required to produce necrosis in Sarcoma 37, an implanted tumor in mice (14), the Spearman rank order coefficient was significant at $p \leqq 0.05$. In another study (11) the ranking of 15 colchicine analogues in producing necrosis in Sarcoma 37 and inhibition of colchicine binding correlated significantly at $p \leqq 0.05$. These correlations support the view that an interaction with tubulin is the basis of the antitumor activity of colchicine and podophyllotoxin. However, regardless of the exact nature of tumor inhibition by these drugs, since the inhibition of colchicine binding does correlate well with this activity, tubulin binding assays *in vitro* may be useful tools in the search for more active antitumor agents.

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