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Podophyllotoxin as a Probe for the Colchicine Binding Site of Tubulin

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The binding of [^{3}H]podophyllotoxin to tubulin, measured by a DEAE-cellulose filter paper method, occurs with an affinity constant of 1.8×10^6 M^{-1} (37° at pH 6.7). Like colchicine, ~0.8 mol of podophyllotoxin are bound per mol of tubulin dimer, and the reaction is entropy-driven (43 cal deg^{-1} mol^{-1}).

At 37° the association rate constant for podophyllotoxin binding is 3.8×10^6 M^{-1} h^{-1}, ~10 times higher than for colchicine; this is reflected in the activation energies for binding which are 14.7 kcal/mol for podophyllotoxin and 20.3 kcal/mol for colchicine. The dissociation rate constant for the tubulin-podophyllotoxin complex is 1.9 h^{-1}, and the affinity constant calculated from the ratio of the rates is close to that obtained by equilibrium measurements.

Podophyllotoxin and colchicine are mutually competitive inhibitors. This can be ascribed to the fact that both compounds have a trimethoxyphenyl ring and analogues of either compound with bulky substituents in their trimethoxyphenyl moiety are unable to inhibit the binding of either of the two ligands.

Tropolone, which inhibits colchicine binding competitively, has no effect on the podophyllotoxin/tubulin reaction. Conversely, podophyllotoxin does not influence tropolone binding. Moreover, the tropolone binding site of tubulin does not show the temperature and pH lability of the colchicine and podophyllotoxin domains, hence this lability can be ascribed to the trimethoxyphenyl binding region of tubulin.

Since podophyllotoxin analogues with a modified B ring do not bind, it is concluded that both podophyllotoxin and colchicine each have at least two points of attachment to tubulin and that they share one of them, the binding region of the trimethoxyphenyl moiety.

Several classes of compounds are thought to interfere with microtubule-dependent cell functions because they bind to tubulin, the subunit of microtubules (1-3). They include colchicine, the *Vinca* alkaloids, such as vinblastine and vincristine, and podophyllotoxin. Although all of these compounds disaggregate microtubules, the *Vinca* alkaloids bind at a site that appears to be unrelated to the colchicine binding site (3-6). On the other hand, podophyllotoxin inhibits colchicine binding competitively (5-10), and this has been ascribed to the fact that both compounds possess a trimethoxyphenyl ring (7). Nevertheless, there are important differences between these ligands. Thus, the tropolone moiety is an attachment point for colchicine to tubulin (7), and podophyllotoxin is devoid of this ring. Secondly, colchicine binds slowly, requiring 1½ h to attain equilibrium at 37° (3, 5-9) and is reversible with difficulty (2, 5, 10), whereas podophyllotoxin is reported to bind and dissociate more rapidly (9, 10). These differences made it difficult to envisage a mechanism for the apparent competition between these two ligands for the binding site and led us to compare some of the kinetic and thermodynamic parameters for their binding as well as to specify the structural requirements that are shared or are different in these two compounds.

EXPERIMENTAL PROCEDURES

Methods

Protein Purification—Tubulin was purified from rat brains in PMG buffer[1] (10 mM sodium phosphate, pH 6.7; 10 mM $MgCl_2$, 10^{-4} M GTP) by the procedure of Weisenberg *et al.* (11), except that DEAE-cellulose was used instead of DEAE-Sephadex. In some experiments we used the polymerization method described by Shelanski *et al.* (12) or a procedure which combined both of the methods: after one or two cycles of polymerization of rat brain tubulin (12), the protein was depolymerized in PMG buffer at 0° for 20 min, applied to a DEAE-cellulose column and eluted in the usual fashion (11). Tubulin thus prepared had the highest colchicine and podophyllotoxin binding activity as judged by the number of moles of ligand bound per mole of protein. The purified protein was stored at −20° in PMG buffer containing 1 M sucrose. The purity of tubulin was checked by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate according to Weber and Osborn (13). Gels contained 5% acrylamide and 0.2% bisacrylamide and were run in phosphate buffer (pH 7.1) at 23°. Overloaded gels yielded a single band.

Protein concentrations were determined by the method of Lowry *et al.* (14), using bovine serum albumin as a standard.

Fluorescence was measured in the thermostated chamber of an Hitachi-Perkin-Elmer MPF-3 spectrofluorometer with excitation and emission wavelengths of 353 and 430 nm for colchicine, and 350 and 410 nm for tropolone.

Incubation Procedure—The incubation medium contained PMG buffer plus tubulin and [^{3}H]podophyllotoxin of known specific activity or other ligands. Tubulin was kept at 0°, and two min before the experiments it was brought to the appropriate temperature. In some experiments, when the tubulin concentration was lower than 1×10^{-7} M, crystalline bovine serum albumin (0.1%) was added. The reaction was stopped by rapidly cooling the tubes to 0°. The binding

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[1] The abbreviations used are: PMG buffer, 10 mM sodium phosphate, 10 mM $MgCl_2$, 10^{-4} M GTP; ANS, 1-anilino-8-naphthalene sulfonate.

assay was performed within 10 min after stopping the reaction.

Colchicine binding was evaluated by the filter disc method of Weisenberg *et al.* (11), modified by Williams and Wolff (15) or by the fluorescence method of Bhattacharyya and Wolff (7). Tropolone binding was determined by fluorescence enhancement occurring in the presence of tubulin (7). Colcemid binding was determined by a method identical to that used for podophyllotoxin (see below).

Podophyllotoxin Binding Assay—The DEAE-filter paper disc assay for colchicine had to be modified to make it suitable for podophyllotoxin. Two DE81 paper discs (Whatman) were washed with cold PMG buffer (4°) by suction, taking care not to dry the paper. Over a period of 1 to 2 min, 100 to 150 μl of the sample were applied and were absorbed to filters. The filters were then rinsed four times with 4 ml of cold PMG buffer by mild suction. The discs were counted in 10 ml of Aquasol (New England Nuclear) or Hydromix (Yorktown) to a counting error of <2%. Identical blanks, lacking only tubulin, amounted to 0.3 to 0.5% of the total radioactivity applied. When 3 or 4 filter papers were used, or when the filters were rinsed with buffer containing 10^{-5} M podophyllotoxin, identical results were obtained. Most of the free podophyllotoxin was removed after the first wash. By contrast, the bound complex remained on the paper and was not washed off with up to seven additional washes as shown in Table I. The formation of podophyllotoxin-tubulin complex, detected by the paper disc method described, is a linear function of protein concentration, as shown in Fig. 1.

The filter method was checked against two other methods. In the first, the comparison was with the Sephadex G-100 column method of Weisenberg *et al.* (11), as shown in Table II. It is clear that the gel filtration and the filter paper methods gave almost identical results; hence for convenience, the latter was used throughout. In the second, a norite/dextran procedure similar to that used by Wilson (10) gave results in excellent agreement with those obtained by the filter disc method. However, the norite method had a high blank (3.5% of total radioactivity), and was not used for this reason.

TABLE I

Quantitation of podophyllotoxin binding by the filter paper method

A sample containing 5×10^{-7} M [^{3}H]podophyllotoxin and 1.8×10^{-6} M tubulin in PMG buffer was incubated for 30 min at 37° and then rapidly cooled at 0°. The sample (100 μl) was applied on two DE81 paper discs and rinsed with 4-ml portions of cold PMG buffer. The blank was prepared by omitting tubulin.

Number of washings	Percent of total counts remaining on filter papers	
	Sample	Blank
0	82.6	74.2
1	56.7	2.7
2	57.6	1.6
3	56.7	0.8
4	61.4	0.6
5	54.3	
8	57.7	0.5

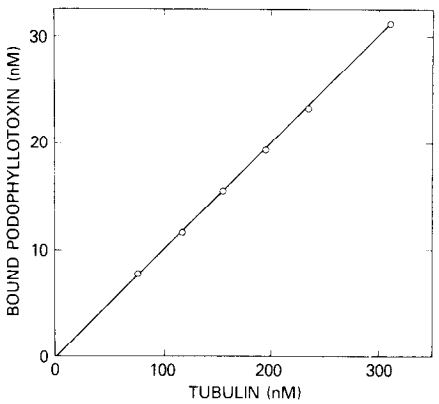


FIG. 1. Linearity of the assay for the podophyllotoxin/tubulin binding reaction. [^{3}H]Podophyllotoxin (8.3×10^{-8} M) was incubated with increasing concentrations of tubulin in PMG buffer (pH 6.7) for 30 min at 37°.

Materials

[^{3}H]Podophyllotoxin, a kind gift of Dr. Martin Flavin (Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md.), had a specific activity of 3.4 Ci/mmol and was labeled in the 4′ position. It was synthesized by methylation of 4′-demethyl podophyllotoxin with [^{3}H]diazomethane (16, 17). The radiochemical purity of [^{3}H]podophyllotoxin was determined by thin layer chromatography on silica gel plates (Quantum Industries Q1F) in four different solvents: (*a*) isopropyl ether:ethanol (19:1), R_F = 0.23; (*b*) chloroform:ethyl acetate (4:1), R_F = 0.31; (*c*) methylene chloride:acetone (4:1), R_F = 0.69; (*d*) ether:methylene chloride (6:1), R_F = 0.24. [^{3}H]Podophyllotoxin was determined on 1-cm sections of the gel scraped into 10 ml of Hydromix (Yorktown). The following purities were found: Solvent *a*, 85.4%; Solvent *b*, 88.5%; Solvent *c*, 91.5%; Solvent *d*, 88.6%.

Tritiated colchicine (ring C, [^{3}H]methoxy), a product of New England Nuclear Corp., had a specific activity of 2.5 Ci/mmol.

Podophyllotoxin was the gift of Dr. W. J. Gensler of Boston University. A Stock solution (1×10^{-2} M) in ethanol was diluted with PMG buffer such that the ethanol concentration was <0.1%. Concentrations of ethanol up to 1% had no detectable effect on the binding reaction. Concentrations were determined from an extinction coefficient of 4400 at 294 nm (16). Guanosine triphosphate (GTP, Grade IIS) and 2-(*N*-morpholino)ethanesulfonic acid were products of Sigma Chemical Co. Tropolone, trimethoxybenzamide and trimethoxybenzaniline were products of Aldrich Chemical Co. and trimethoxyphenylpropionic acid was a product of K & K. Tropolone methyl ether was synthesized with diazomethane according to published procedures (18, 19). Colchicine analogues were gifts of Dr. Colin Chignell. Vinblastine sulfate was a gift from Eli Lilly and Co. 4′-Demethyl deoxypodophyllotoxin-β-D-glucoside and 4′-carbobenzoxy-4′-demethyl podophyllotoxin were the kind gifts of Dr. A. von Wartburg (Pharmaceutical Division, Sandoz Ltd., Basel, Switzerland).

RESULTS

Binding Conditions—Preliminary experiments had shown that the binding of podophyllotoxin to tubulin was relatively fast and, in the range of concentrations used in most experiments (10^{-5} to 10^{-7} M podophyllotoxin and 10^{-6} M tubulin), at 37° less than 20 min were needed to reach equilibrium. For this reason, samples were generally incubated for 20 to 30 min and then analyzed.

The optimum pH for binding was 6.6 to 6.9 (Fig. 2); Tris/HCl and sodium phosphate buffer of the same pH gave similar results. The pH curve of podophyllotoxin binding does not differ substantially from that of colchicine binding to tubulin (5, 20).

No binding could be detected when podophyllotoxin was incubated with the following proteins: bovine serum albumin, rabbit muscle aldolase, bovine pancreatic ribonuclease, horse liver alcohol dehydrogenase, trypsin, beef liver catalase, beef

TABLE II

Determination of podophyllotoxin binding to tubulin by the gel filtration and the filter paper methods

Three samples containing podophyllotoxin and tubulin in the concentrations stated were incubated at 37° for 20 min in PMG buffer (400 μl total volume), rapidly cooled at 0°, and then analyzed by the gel filtration method (11) and by the filter paper method. In the gel filtration assay, 100 μl of the incubated samples were applied to a Sephadex G-100 column (1 × 15 cm), eluted with PMG buffer, and 1-ml fractions collected (11). The recovery of the column was equal to 94 to 97%. The filter paper assay was performed as stated in the text.

[Podophyllotoxin]	[Tubulin]	[Bound podophyllotoxin]	
		Filter paper	Gel filtration
μM		μM	
5.0	2.4	0.96	1.03
7.5	2.4	1.15	1.13
10.0	2.4	1.27	1.21

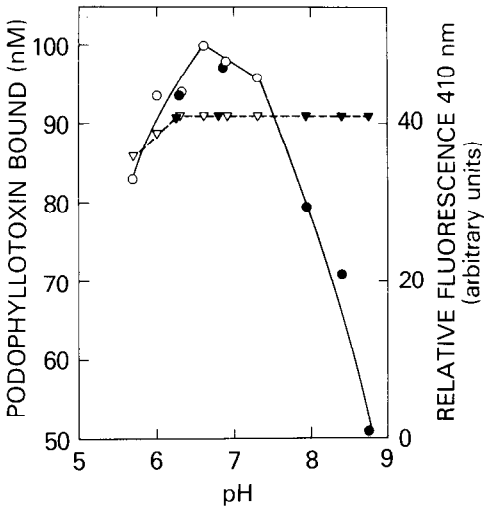


FIG. 2. pH dependence of podophyllotoxin and tropolone binding to tubulin. [^{3}H]Podophyllotoxin (3.9×10^{-7} M) was incubated with tubulin (1.3×10^{-6} M) for 20 min at 37° in 10 mM $MgCl_2$ and either 10 mM sodium phosphate buffer (○) or 10 mM Tris/HCl (●). Tropolone (3.3×10^{-5} M) was incubated for 20 min with tubulin (2.1×10^{-6} M) at 37° in 10 mM $MgCl_2$ and 10 mM sodium phosphate buffer (△) or 10 mM Tris/HCl (▲). Tropolone binding was evaluated by measuring enhancement of fluorescence.

liver glutamic dehydrogenase, rabbit muscle lactic dehydrogenase, and bovine thyroglobulin.

No chemical modification of the ligand could be detected upon complex formation. Tubulin was incubated for 30 min with [^{3}H]podophyllotoxin and the complex was separated by the DEAE-filter paper disc method. The podophyllotoxin was then released from tubulin by heating the DEAE-paper at 60° for 15 min in water. The solution was concentrated and chromatographed on silica gel plates in the four different solvents listed under "Experimental Procedures." In all solvents, more than 97% of the radioactivity moved with authentic podophyllotoxin.

Loss of Binding Activity of Tubulin—At 37°, the colchicine binding activity of uncomplexed tubulin decays in a first order manner with a $t_{1/2}$ of 3 to 5 h, and vinblastine and sucrose protect this binding activity (5, 8, 21–23). In Fig. 3, we compare the effect of preincubation of tubulin at 37° on the binding capacity of the protein toward colchicine and podophyllotoxin. The decay of the binding capacity for colcemid was also tested. The $t_{1/2}$ for podophyllotoxin was 5.0 h, for colchicine it was 4.5 h, and for colcemid it was 5.8 h. When the incubation time is added, these $t_{1/2}$ values become, respectively, 5.5 h, 6.0 h, and 6.3 h.

When the preincubation was carried out in the presence of vinblastine or sucrose, the decay of the binding activity for all ligands was much slower ($t_{1/2} > 14$ h in all cases). Vinblastine afforded better protection than sucrose. Heating the protein at temperatures higher than 37° accelerated the decay of the binding activity, and at 60° less than 15 min were needed to abolish completely the ability of tubulin to react with podophyllotoxin or colchicine (Table III).

These results suggested that the lability of colchicine binding resided at least in the trimethoxyphenyl portion of the binding site. They did not, however, shed light on the thermal properties of the tropolone portion of the colchicine site. To investigate this question, we measured the decay in the ability of tubulin to enhance the fluorescence of tropolone. As shown in Table III, heating of tubulin left the tropolone binding

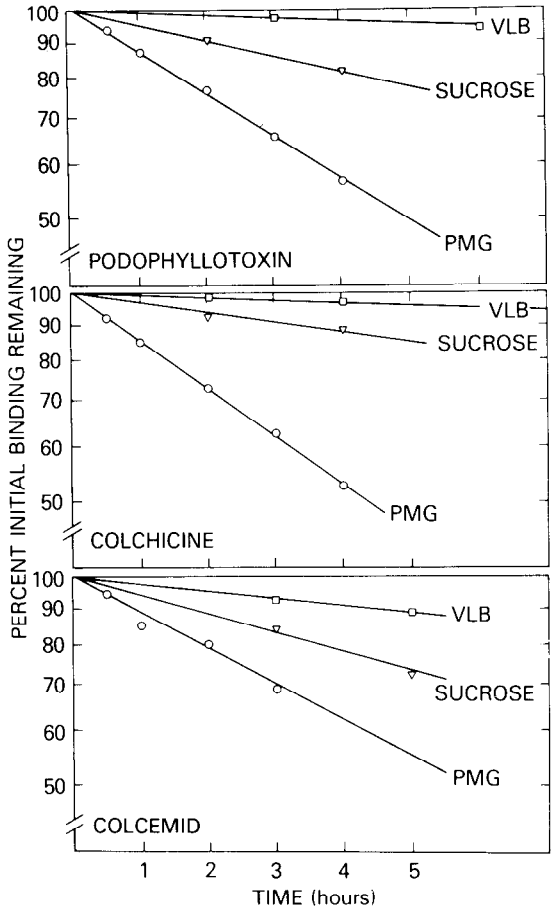


FIG. 3. Podophyllotoxin, colchicine, and colcemid binding to tubulin as a function of preincubation time at 37°. Tubulin (5.6×10^{-7} M) was preincubated at 37° in PMG buffer (○——○), PMG buffer containing 1 M sucrose (△——△), PMG buffer containing 1×10^{-4} M vinblastine (*VLB*) (□——□), all at pH 6.7. After the preincubation period the binding activity of tubulin was measured by incubating an aliquot of the sample with the ^{3}H-labeled ligands at 37° for 30 min (colcemid and podophyllotoxin), and for 1 h 30 min in the case of colchicine. Colcemid binding was assayed by a method identical to that used for podophyllotoxin.

capacity of the protein completely intact whereas colchicine and podophyllotoxin binding were completely abolished. An additional difference between the trimethoxyphenyl and tropolone portions of the colchicine binding site is demonstrated in the pH profile shown in Fig. 2. While podophyllotoxin shows a rather sharp optimum near pH 6.7, the fluorescence of tropolone was unaffected over a pH range of 6.5 to 8.8. It seems clear, therefore, that the high lability of the colchicine binding site is restricted to the trimethoxyphenyl portion.

Binding Parameters at Equilibrium—If the overall binding reaction is

$$T + nP \rightleftharpoons TP_n \quad (1)$$

where T is tubulin, P is podophyllotoxin, and n is the number of podophyllotoxin binding sites on tubulin, then at equilibrium

$$r = \frac{nKP}{1 + KP} \quad (2)$$

where, r is the number of moles of podophyllotoxin bound per mole of tubulin, K is the affinity constant $\left(K = \frac{[TP_n]}{[P]^n[T]}\right)$.

Rearrangement of Equation 2 gives

$$r/P = nK - Kr, \quad (3)$$

which is the basis of the Scatchard plot shown in Fig. 4. The number of podophyllotoxin binding sites per tubulin dimer was equal to 0.71 (Fig. 4). The [^{3}H]podophyllotoxin obtained from the complex was 97% pure whereas the mean radiochemical purity of the starting material was 89% (see "Methods"). Thus the impurities did not appear to bind to tubulin, and the corrected value for the stoichiometry is 0.8 mol of podophyllotoxin per mol of tubulin (110,000 daltons). This is identical to the stoichiometry reported for colchicine (3, 6, 7).

The affinity constant at 37° was 1.6 to 1.8 × 10^6 M^{-1} (K_d = $1/K$ = 5.5 to 5.2 × 10^{-7} M). This should be compared with a K_d value of 7 × 10^{-7} M obtained for pig brain tubulin (9), and a concentration of 5 × 10^{-7} M required for half-maximal inhibition of axonal transport (24). At lower temperatures, the constant was lower (Fig. 4). A van't Hoff plot of log K *versus* the reciprocal of the absolute temperature was linear in the region 22–37° (*inset* Fig. 4) and yielded an estimate of 4.8 kcal/mol for the standard enthalpy of binding (ΔH^0). The standard free energy (ΔG^0) of binding at 37° was equal to −8.8 kcal/mol and ΔS^0 = 43 cal deg^{-1} mol^{-1}. The entropy value should be compared with 62 cal deg^{-1} mol^{-1} obtained from equilibrium binding of colchicine (7).

Association Rate Constant and Activation Energy – If podophyllotoxin binding is assumed to be bimolecular, the association rate constant (k_1) is

$$k_1 = \frac{d[PT]}{dt}/[P][T] \quad (4)$$

where $(d[PT])/dt$ is the rate of formation of the complex and $[P]$ and $[T]$ are the concentrations of free podophyllotoxin and unoccupied tubulin. 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 $P = P_0$ and $T = T_0$, where P_0 and T_0 are the initial concentrations.

When the protein concentration was 190 nM, the rate curves were linear for the first 2 to 3 min of incubation over a concentration of 58 to 188 nM podophyllotoxin (Fig. 5). Similarly, when podophyllotoxin was held constant at a 138 nM

TABLE III

Temperature effect on colchicine, podophyllotoxin, and tropolone binding activity of tubulin

Tubulin (1.5 × 10^{-6} M) was preincubated in PMG buffer, pH 6.7, at the temperature and for the time indicated. Then 200 μl of this sample were added to 50 μl of solution containing 5.8 × 10^{-7} M [^{3}H]podophyllotoxin, 1.2 × 10^{-6} M colchicine, or 10^{-5} M tropolone, and were incubated for 30 min at 37°. Podophyllotoxin binding was measured by the disc method, whereas colchicine and tropolone binding were measured by fluorescence (7). The results are expressed as the percentage of the binding detected in samples when the protein was not preincubated.

Temperature	Time	Percentage of initial binding activity		
		Colchicine	Podophyllotoxin	Tropolone
	h			
37°	1	82	86	98
45	1	60	46	97
60	0.5	0	0	98

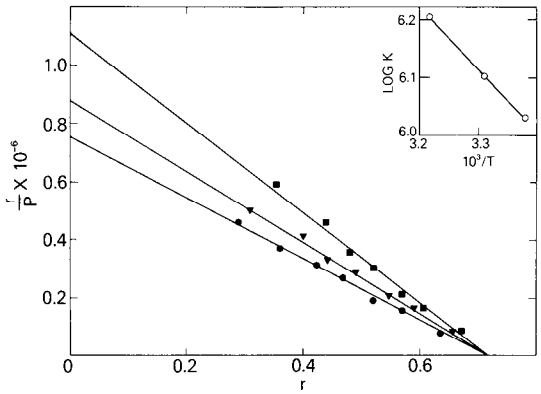


FIG. 4. The temperature dependence of podophyllotoxin binding to tubulin. The reaction mixture contained 5.8 × 10^{-7} M tubulin in PMG buffer, pH 6.7. Tubes were incubated for 20 min at 37° (■——■), 40 min at 28° (▲——▲), and 60 min at 22° (●——●). Results are expressed by a Scatchard plot (r = moles of podophyllotoxin bound per mole of tubulin (110,000 daltons), P = free podophyllotoxin concentration). The *inset* shows log K against $10^3/T$.

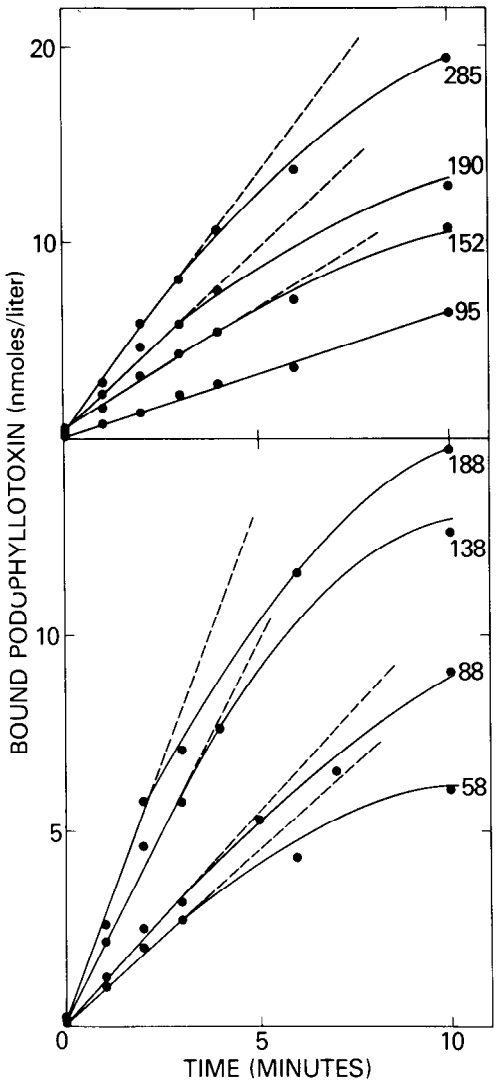


FIG. 5. The rate of podophyllotoxin binding as a function of the concentrations of podophyllotoxin and tubulin. All incubations were at 37° in PMG buffer. *Upper panel*, podophyllotoxin was constant at 138 nM. *Numbers* refer to nM tubulin concentrations. *Lower panel*, tubulin was held constant at 190 nM. Numbers refer to nM podophyllotoxin concentrations. The *dashed lines* are continuations of the slopes for the initial rates.

concentration, binding curves remained linear for 3 to 10 min over tubulin concentrations ranging from 95 to 285 nM. The second order rate constants (k_1) calculated from these data are listed in Table IV; the mean of these values is 3.8×10^6 M^{-1} h^{-1}. These results suggest that podophyllotoxin binding is truly second order under the conditions tested.

Since the association rate constant determined for podophyllotoxin is 10 times higher than that for colchicine (0.36×10^6 M^{-1} h^{-1} (21), 0.41×10^6 M^{-1} h^{-1} (25), or 0.12 to 0.48×10^6 M^{-1} h^{-1} (26)), it was important to measure the activation energies (E_a) for both reactions. A comparison of the temperature effect on colchicine and podophyllotoxin binding, and the Arrhenius plots derived therefrom, is presented in Fig. 6. The activation energy for podophyllotoxin is 14.7 kcal/mol, that for colchicine 20.3 kcal/mol. The higher activation energy for colchicine binding explains, at least in part, its lower association rate.

Dissociation Rate Constant (k_{-1})—In view of the discrepancy between the affinity constant (K) determined in equilibrium experiments and the ratio of the association (k_1) and dissociation (k_{-1}) rate constants for colchicine (21, 25, 26), it was of interest to determine the k_1/k_{-1} ratio for podophyllotoxin binding. In order to determine the dissociation of the podophyllotoxin-tubulin complex, the bound podophyllotoxin was separated from the free by passing the sample at 4° through a Sephadex G-75 column (24 × 1.4 cm). The podophyllotoxin-tubulin complex was diluted ~60 times so that reassociation was negligible, and was then incubated at 37° in PMG buffer. As shown in Fig. 7, the $t_{1/2}$ of dissociation of the complex was ~22 min, giving $k_{-1} = 1.9$ h^{-1}. The ratio of the rate constant yields an association constant of 2.1×10^6 M^{-1}, which is in good agreement with the value obtained by equilibrium methods.[2]

Analogues—Numerous colchicine analogues have been found to block [^{3}H]colchicine binding to tubulin (2, 7, 8). In most cases it has not been established whether these compete for the A ring or C ring regions or both, or whether binding at one locus necessarily implied occupancy of the whole site. We therefore tested certain analogues for their effect on podophyllotoxin binding on the assumption that only the trimethoxyphenyl moiety of this compound was recognized by the colchicine binding site. If the trimethoxyphenyl ring is a site of interaction of both colchicine and podophyllotoxin with tubulin, it would be expected that analogues with bulky substituents in the trimethoxyphenyl ring are unable to bind to tubulin. This is indeed the case: colchicoside, in which one methoxy group in colchicine ring A is replaced by a sugar, and 4′-demethyl deoxypodophyllotoxin-β-D-glucoside and 4′-carbobenzoxy-4′-demethyl podophyllotoxin, which possess bulky substituents in the 4′ position of the podophyllotoxin molecule, are totally ineffective in inhibiting colchicine or podophyllotoxin binding (Table V). These analogues also supply evidence that podophyllotoxin binds to tubulin through its trimethoxyphenyl portion. Simple trimethoxyphenyl derivatives do not appear to interact with the colchicine binding site (Table V). However, it has recently been shown that mescaline (3,4,5-trimethoxyphenethylamine) is an antimitotic agent (27).

Since podophyllotoxin is known to inhibit colchicine binding competitively, it was expected that colchicine would similarly block podophyllotoxin binding comparatively. Rearrangement of Equation 2 yields

$$1/r = 1/n + \left(\frac{1}{nK}\right)\frac{1}{P} \tag{5}$$

In the presence of an inhibitor, I, Equation 5 becomes

$$1/r = 1/n + \left(\frac{1 + I/K_i}{nK}\right)\cdot\frac{1}{P} \tag{6}$$

where $K_i = \frac{[T][I]}{[TI]}$. As shown in Fig. 8, colchicine was a competitive inhibitor of podophyllotoxin binding. The K_I was 2.1×10^{-6} M. An important problem for which we have been unable to supply an answer is the discrepancy in ligand affinities for tubulin when these are measured as the K_i or K_d under identical conditions. The K_i values for podophyllotoxin and colchicine are 2.0×10^{-6} M and 2.1×10^{-6} M, respectively (7). These differences have been consistently observed. It is conceivable that these discrepancies result from incomplete equilibration. However, as shown by others as well as by us (3, 8, 25, 28), these reactions were carried out at a time when >90% equilibrium had been attained. Furthermore, since podophyllotoxin binding is more rapid than colchicine binding, failure of equilibration would have opposing effects on K_i and K_d depending on which ligand is labeled. One possible explanation, that the two ligands might interact, seems unlikely since no difference spectra could be elicited between combined and separate solutions of podophyllotoxin and colchicine, and since the mobility of 1×10^{-6} M labeled podophyllotoxin on Sephadex G-10 (in PMG buffer) was not altered by 1×10^{-5} M colchicine. It is possible that the discrepancy between the two constants results from the partial overlap of the binding sites.

Surprisingly, manipulations of the tropolone ring, as shown in isocolchicine, produced analogues that were not able to block podophyllotoxin binding. On the other hand, although both tropolone and methyltropolone bind to the site, as shown by enhancement of fluorescence or blocking of colchicine binding (7), neither has any effect on podophyllotoxin binding. This suggests that the remainder of the podophyllotoxin molecule need not overlap the tropolone domain of the colchicine binding site when the trimethoxyphenyl moiety is binding. This is also shown by the finding that podophyllotoxin did not affect the fluorescence enhancement of tropolone resulting from its binding to tubulin.

TABLE IV

Association rate constants determined at different podophyllotoxin and tubulin concentration

[^{3}H]Podophyllotoxin and tubulin were incubated at the concentrations stated in the table at 37° and the reaction rate ($d[PT]/dt$) determined in the linear part of the plot of complex formation over time (Fig. 5). The association rate constant, k_1, was calculated as stated in the text.

[Podophyllotoxin]	[Tubulin]	$d[PT]/dt$	k_1
nM		*nmol* l^{-1} h^{-1}	10^6 M^{-1} h^{-1}
58	190	45	4.1
88	190	60	3.6
138	190	110	4.2
188	190	168	4.7
138	95	37	2.8
138	152	73	3.5
138	285	150	3.8

[2] For colchicine, the apparent dissociation is the sum of decay of the site plus true dissociation (22). Since $t_{1/2}$ for the podophyllotoxin-tubulin complex dissociation is 22 min and decay of unoccupied site is about 300 min, the decay factor does not play a significant role in podophyllotoxin dissociation.

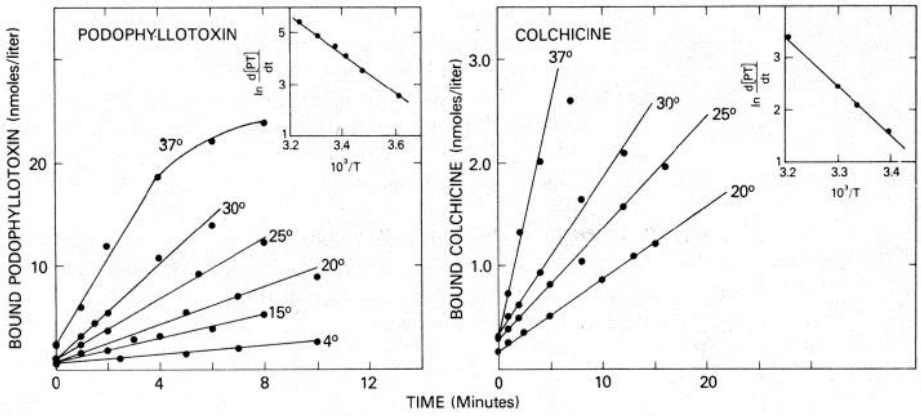


FIG. 6. Temperature dependence of podophyllotoxin and colchicine association rates. All reactions were carried out in PMG buffer. *Left panel*, 82 nM podophyllotoxin was incubated with 1.62 μM tubulin in PMG buffer. The *inset* depicts the rates plotted as $(d[PT])/dt$ *versus* $10^3/T$, where $(d[PT])/dt$ is the rate of formation of the podophyllotoxin-tubulin complex expressed as nmol liter^{-1} h^{-1}. *Right panel*, 84 nM colchicine was incubated with 2.14 μM tubulin in PMG buffer. The *inset* shows a plot of $(d[CT])/dt$ *versus* $10^3/T$, where $(d[CT])/dt$ is the rate of formation of the colchicine-tubulin complex expressed as nmol liter^{-1} h^{-1}.

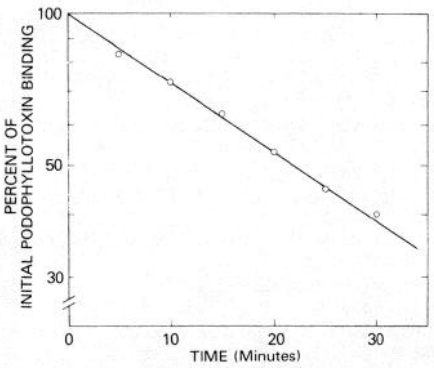


FIG. 7. Dissociation of the tubulin-podophyllotoxin complex. Tubulin (1.3×10^{-5} M) was incubated with [^{3}H]podophyllotoxin (7.3×10^{-7} M) for 30 min at 37° and the bound podophyllotoxin was separated from the free by passing the sample through a Sephadex G-75 column (24 × 1.4 cm) equilibrated with PMG buffer at 4°. The podophyllotoxin · tubulin complex, eluted in the void volume, was diluted ~60 times with PMG buffer containing 0.2% albumin and then incubated at 37°.

DISCUSSION

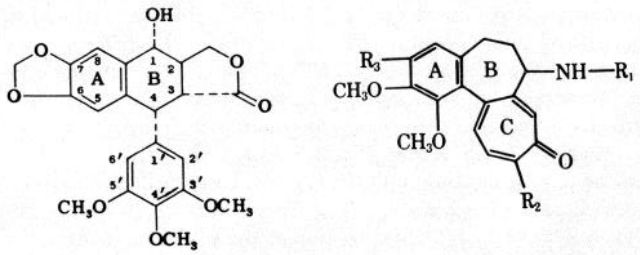
A competitive inhibitor is usually considered to be a compound that prevents "substrate" binding by an interaction at the same site. However, competitive kinetics are also possible by mutual distortion of the respective binding sites or by steric hindrance from adjacent sites (29). These variations are not easily distinguished from one another. It was, therefore, important to characterize the podophyllotoxin effect since only a small portion of its structure resembles colchicine. The following properties of the two binding processes were found to be very similar or identical. (*a*) A pH optimum at 6.7. The narrow range of pH optimum reflects the ionization of the protein since neither colchicine or podophyllotoxin possess ionizable groups. (*b*) Stoichiometry approaching 1 mol of ligand per 110,000 molecular weight. The observed value for podophyllotoxin, corrected for impurities, was ~0.8 with a podophyllotoxin/colchicine ratio of ~1.0 (*c*) Lability. The site decays with a half-life of 4 to 6 h for both ligands. Vinblastine or 1 M sucrose stabilize the binding of both ligands identically. (*d*) Similar thermodynamic parameters at equilibrium. Both reactions are entropy-driven: 62 cal deg^{-1} mol^{-1} for colchicine and 43 cal deg^{-1} mol^{-1} for podophyllotoxin. (*e*) Mutual competitive inhibition. Thus colchicine blocks podophyllotoxin binding and podophyllotoxin blocks colchicine binding.

The above arguments suggest that these two ligands share at least a part of the same binding site on tubulin. Nevertheless, certain differences in the binding processes for these

TABLE V

Comparison of the effect of colchicine analogues on podophyllotoxin and colchicine binding to tubulin

All incubations were for 1.0 h at 37°. For the binding of podophyllotoxin, concentrations were 1.5×10^{-7} M [^{3}H]podophyllotoxin, 2.7×10^{-6} M tubulin, and 2.5×10^{-4} M for all the analogues, except the podophyllotoxin derivatives for which the concentrations were 4.3×10^{-8} M podophyllotoxin, 1.3×10^{-6} M tubulin, and 8.3×10^{-6} M analogue. This was necessary in order to maintain a higher molar ratio with these poorly soluble compounds. The colchicine concentrations were 1.6×10^{-7} M for the trimethoxyphenyl analogues and 3×10^{-8} M for the podophyllotoxin derivatives with analogue concentrations as above. The plus (+) indicates that the podophyllotoxin or colchicine binding was <9% than that of a control sample, the minus (−) means that binding was >98% of that of the control.



Compound	Colchicine substitutions			Inhibition of	
	R_3	R_2	R_1	Podophyllotoxin binding	Colchicine binding
Colchicine	OCH_3	OCH_3	$COCH_3$	+	+
Colcemid	OCH_3	OCH_3	CH_3	+	+
Isocolchicine	OCH_3	—[a]	$COCH_3$	−	−
Colchicoside	$C_6H_{11}O_9$	OCH_3	$COCH_3$	−	−
Podophyllotoxin				+	+
Trimethoxybenzamide				−	−
Trimethoxybenzaniline				−	−
Trimethoxyphenylpropionic Acid				−	−
4′-Carbobenzoxy-4′-demethyl podophyllotoxin				−	−
4′-Demethyl desoxypodophyllotoxin-β-D-glucoside				−	−
Tropolone				−	+
Methyltropolone				−	+

[a] In isocolchicine ring C, the oxygen and R_2 group (OCH_3) are inverted with respect to colchicine.

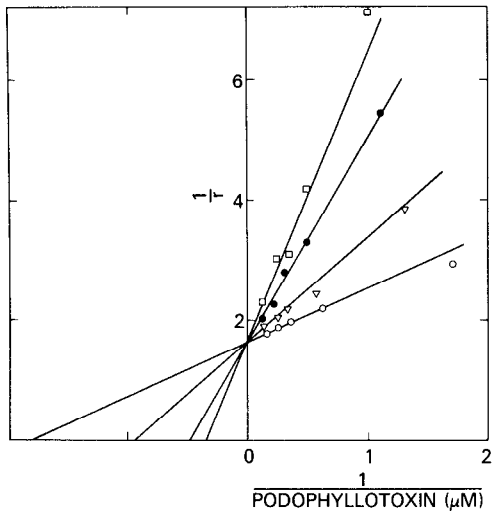


FIG. 8. Double reciprocal plot of colchicine inhibition of podophyllotoxin binding. The concentrations of colchicine used were 2.5 μM (△——△), 5.0 μM (●——●), 7.5 μM (□——□). Open circles (○——○), no colchicine. The tubulin concentration was 1.9 μM, temperature = 37°, incubation time = 1.5 h; r is the number of moles of podophyllotoxin bound per mole of tubulin.

compounds exist. These are the rate of binding and the effect of tropolone on binding.

Colchicine and podophyllotoxin bind relativcly slowly when compared with ANS, which interacts "instantaneously" with tubulin and at a different site (30, 31). However, podophyllotoxin is a faster reactant than colchicine, and at 37°, its forward rate constant (k_1) is 10 times higher than that for colchicine. This appears to be mainly due to the difference in the activation energy which is 20.3 kcal/mol for colchicine and 14.7 kcal/mol for podophyllotoxin. The structural reason for this difference is, at present, unknown.

Tropolone inhibits colchicine binding competitively (7), but has no effect on podophyllotoxin. Conversely, podophyllotoxin does not affect tropolone binding. The results strongly suggest that the trimethoxyphenyl portion of the colchicine domain is shared by colchicine and podophyllotoxin, whereas the tropolone portion of the site recognizes only colchicine and not podophyllotoxin. Interestingly enough, the tropolone site does not show the pH and temperature dependence exhibited by podophyllotoxin or colchicine, thus suggesting that these properties are referable to the trimethoxyphenyl binding region. That the trimethoxyphenyl ring is a point of attachment of both colchicine and podophyllotoxin is shown by the observation that analogues with a bulky substituent instead of one of the OCH_3 groups are unable to inhibit colchicine or podophyllotoxin binding.

Surprisingly, colchicine analogues with a modified C ring and an intact A ring are unable to inhibit podophyllotoxin binding (Table V). These observations suggest that the colchicine molecule is very rigid, such that when the tropolone moiety cannot bind the trimethoxyphenyl portion is sterically hindered from approaching its portion of the binding site. By the same token, if the A ring is hindered then the C ring is prevented from binding as shown by the absence of fluorescence in colchicoside (7). This is in agreement with the crystallographic data for colchicine (32) and colcemid (33).

Like colchicine, podophyllotoxin must also have at least one other binding site to tubulin. This is demonstrated by the marked differences in biological activity of stereoisomers of podophyllotoxin *e.g. cis-trans* isomers in the B ring of podophyllotoxin (16). Similarly, succinylation of the 1-OH position abolishes the binding activity of podophyllotoxin (9). It remains to be determined whether the tetrahydronaphthol moiety alone will block podophyllotoxin binding and not colchicine binding.

We may conclude, therefore, that colchicine and podophyllotoxin each bind at least at two tubulin domains and have one of these in common, that portion of the site where the trimethoxyphenyl ring interacts.

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