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Mechanism of Colchicine Binding to Tubulin. Tolerance of Substituents in Ring C′ of Biphenyl Analogues[†,‡]

José M. Andreu,[§] Marina J. Gorbunoff,[∥] F. Javier Medrano,[§] Miriam Rossi,[⊥] and Serge N. Timasheff*[,∥]

Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, 28006 Madrid, Spain, Department of Chemistry, Vassar College, Poughkeepsie, New York 12601, and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

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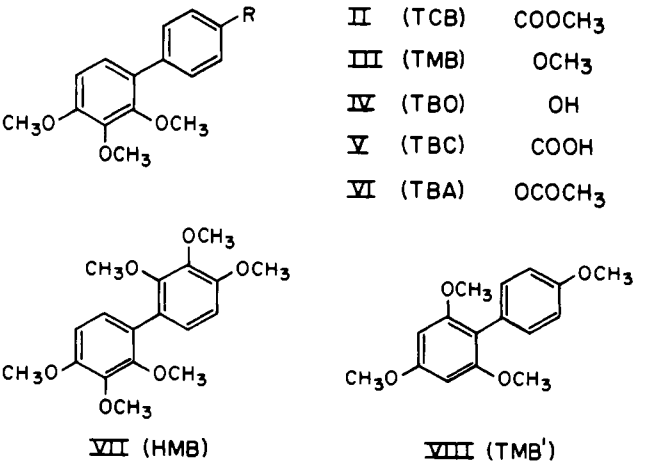
ABSTRACT: The limits of structural variation of the substituent in position 4′ of ring C′ of biphenyl colchicine analogues (ring C in colchicine) were probed by the synthesis of a number of analogues and the examination of their binding to tubulin and its consequences. Binding was found to require the location in three-dimensional space of the oxygen in the 4′-substituent at a locus not far distant from those of the colchicine ring C oxygens. All those analogues that bind to the colchicine site of tubulin induced the GTPase activity and inhibited microtubule assembly, those containing a carbonyl group substoichiometrically and the others stoichiometrically. A similar relation was found for the induction of the abnormal polymerization of the colchicine analogue–tubulin complex, with methoxy-containing compounds requiring a higher temperature to induce the polymerization. A concerted analysis of the binding thermodynamics of colchicine and its various analogues has shown full consistency with the previously proposed two-step binding pathway that involves two nonidentical binding moieties in the ligand [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 534–543]. Comparison of the binding parameters of colchicine, its des(ring B) analogue (MTC), and ring A and C compounds individually with the thermodynamic parameters deduced for the first steps of the bindings of colchicine and MTC [Engelborghs, Y., & Fitzgerald, T. J. (1987) *J. Biol. Chem. 262*, 5204–5209] have led to the conclusion that binding can occur by two pathways leading to the identical product. In the first pathway, ring A binds first; this is followed by a rate-determining thermodynamically indifferent reaction (protein conformation change), and finally a rapid binding of ring C. In the second pathway, the events are the same except that the order of binding of the rings is reversed. Colchicine, due to the steric hindrance of ring B, can follow only the second pathway. For MTC, both kinetic pathways are open and binding may be initiated by random first contact of either ring A or ring C.

The finding that the binding pattern of biphenyl colchicine analogues was controlled by the nature of the oxygen-containing group in position 4′ of ring C′, carbonyl or ether, led us to investigate the limits of variation in this substituent that would still be compatible with binding into the colchicine site on tubulin. The similarity to the binding pattern of allocolchicine diminished in this order: carbonyl (TKB)[1] (structure I, Chart I) > carbomethoxy (TCB, structure II) > methoxy (TMB, structure III), in position 4′ (Medrano et al., 1991). As a result, the substituent in this position was varied further, and the effects on binding to tubulin were examined. The new compounds included 2,3,4-trimethoxy-1,1′-biphenyl-4′-ol (TBO, structure IV), the phenol derivative of TMB, 2,3,4-trimethoxy-1-1′-biphenyl-4′-carboxylic acid (TBC, structure V), the free acid derivative of TCB, and

Chart I



VII (HMB) VIII (TMB′)

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[‡] This paper and the preceding one are dedicated to Professor Robert H. Abeles on the occasion of his 65th birthday.

* To whom correspondence should be addressed.
[§] Centro de Investigaciones Biologicas.
[∥] Brandeis University.
[⊥] Vassar College.

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2,3,4-trimethoxy-1,1′-biphenyl-4′-acetate (TBA, structure VI), the TCB analogue with the ester reversed in position 4′. Two further analogues were 2,2′,3,3′,4,4′-hexamethoxy-1,1′-biphenyl (HMB, structure VII), which is both a dimer of ring A and a TMB analogue with positions 2′ and 3′ of ring C methoxylated, and 2,4,4′,6-tetramethoxy-1,1′-biphenyl (TMB′, structure VIII), which is a TMB analogue with the ring A methoxys positioned symmetrically. The results of these studies and comparison with previously described analogues are presented in this paper, as well as a concerted model of binding to the colchicine site on tubulin.

Materials and Methods

Ligands and Other Materials. Colchicine and podophyllotoxin were from Aldrich Chemical Co. 2-Methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) was a gift from Dr. T. J. Fitzgerald (Fitzgerald, 1976). GTP dilithium salt was from Boehringer-Mannheim. [γ-^{32}P]GTP, triethylammonium salt, was from Amersham (>10 Ci/mmol). Sephacryl S-300 was from Pharmacia. Cocktail-22 Normascint scintillation liquid was from Scharlau. Ethylenebis(oxyethylenenitrilo)tetraacetic acid and sodium dodecyl sulfate were from Sigma. Glycerol was from Merck, analytical grade, and all other chemicals were of reagent grade. The synthesis of the ligands is described below. To check their purity, the products (TBO, TBC, TBA, HMB, and TMB′) were subjected to thin-layer chromatography on 0.25-mm-thick silica gel plates (Fertigplattin, Merck) developed with chloroform–acetone–diethylamine (7:2:1) or dichloromethane–methanol (99:1). All the ligands gave a single spot under ultraviolet light. The ligands were dissolved in dimethyl sulfoxide and stored at −20 °C. The residual dimethyl sulfoxide in the experiments was less than 1%, and the ligands could be dissolved to (3–5) × 10^{-4} M in PG buffer. The concentrations of the ligands were measured spectrophotometrically. The extinction coefficients were determined by weighing dry crystals of the compounds with a Mettler UM-3 electrobalance, dissolving them gravimetrically in a final solution of PG buffer, and recording the UV spectrum. Four independent determinations gave ϵ_{256nm} = 15 900 ± 200 M^{-1} cm^{-1} for TBO, ϵ_{272nm} = 18 000 ± 150 M^{-1} cm^{-1} for TBC, ϵ_{255nm} = 14 400 ± 220 M^{-1} cm^{-1} for TBA, ϵ_{248nm} = 12 700 ± 90 M^{-1} cm^{-1} for HMB, and ϵ_{251nm} = 10 800 ± 200 M^{-1} cm^{-1} for TMB′.

Synthesis of Ligands. The following compounds were prepared as described previously from 1,2,3-trimethoxy-4-iodobenzene and the respective 4-iodo-substituted phenyl (Medrano et al., 1989).

(1) 2,2′,3,3′,4,4′-Hexamethoxy-1,1′-biphenyl (HMB). Mp: 122.0–125.0 °C (lit. mp 123 °C) (*Beilstein* E III 6, p 6951). Anal. Found: C, 64.65; H, 6.63. Calcd: C, 64.50; H, 6.67. MS: MW 334 (theory 334). UV: λ_{max} 247.0 nm (15% CH_3OH–H_2O). IR (CCl_4): 1596 (aromatic); 1462 (aromatic); 1220 (C–O); 1096 (C–O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 6.94 [d, J = 8.4 Hz, H-C(5), H-C(5′)]; 6.71 [d, J = 8.4 Hz, H-C(6), H-C(6′)]; 3.92 (s, 2 m-OCH_3); 3.90 (s, 2 p-OCH_3); 3.70 (2 o-OCH_3). ^{13}C NMR ($CDCl_3$): δ 152.9 (C-2, C-2′); 151.7 (C-4, C-4′); 142.0 (C-3, C-3′); 125.4 (C-6, C-6′); 125.1 (C-1, C-1′); 106.7 (C-5, C-5′); 60.8 (2 OCH_3); 60.7 (2 OCH_3); 55.9 (2 OCH_3).

(2) 2,3,4-Trimethoxy-1,1′-biphenyl-4′-acetate (TBA). MP: 61.5–62.0 °C. Anal. Found: C, 67.46; H, 6.32. Calcd: C, 67.53; H, 6.00. MS: MW 302 (theory 302). UV: λ_{max} 255.0 nm (15% CH_3OH–H_2O). IR (CCl_4): 1680 (C=O); 1602 (aromatic); 1358 (ester CH_3); 1263 (C–O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.52 [d, J = 8.7 Hz, H-C(2′), H-C(6′)]; 7.13 [d, J = 9.0 Hz, H-C(3′), H-C(5′); 7.03 [d, J = 8.7 Hz, H-C(6)]; 6.75 [d, J = 8.7 Hz, H-C(5)]; 3.94 (s, ring A m-OCH_3); 3.91 (s, ring A p-OCH_3); 3.68 (s, ring A o-OCH_3); 2.33 (s, ester CH_3). ^{13}C NMR ($CDCL_3$): δ 169.5 (C=O); 153.2 (C-2); 151.3 (C-4); 149.5 (C-4′); 142.5 (C-3); 135.7 (C-1′); 130.0 (C-2′, C-6′); 127.7 (C-1); 124.7 (C-6); 121.1 (C-3′, C-5′); 107.5 (C-5); 60.9 (2 ring A OCH_3); 56.0 (ring A OCH_3); 21.1 (ester OCH_3).

(3) 2,4,4′,6-Tetramethoxy-1,1′-biphenyl (TMB′). TMB′ was prepared from 2,4,6-trimethoxy-4-iodobenzene and 4-iodoanisole. MP: 104.0 °C (lit. mp 102–103 °C) (*Beilstein* E IV 6, p 7743). Anal. Found: C, 69.96; H, 6.72. Calcd: C, 70.05; H, 6.61. MS: MW 274 (theory 274). UV: λ_{max} 250.0 nm (15% CH_3OH–H_2O). IR (CCl_4): 1590 (aromatic); 1462 (aromatic); 1226 (C–O); 1148 (C–O) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.28 [d, J = 9.0 Hz, H-C(2′), H-C(6′)]; 6.95 [d, J = 8.7 Hz, H-C(3′), H-C(5′)]; 6.25 [s, H-C(3), H-C(5)]; 3.88 (s, ring A p-OCH_3); 3.85 (s, ring C OCH_3); 3.74 (s, 2 ring A o-CH_3). ^{13}C NMR ($CDCl_3$): δ 160.2 (C-4); 158.4 (C-2, C-6); 158.0 (C-4′); 132.1 (C-2′, C-6′); 126.1 (C-1′); 113.2 (C-3′, C-5′); 112.0 (C-1); 90.8 (C-3, C-5); 55.8 (ring A OCH_3); 55.3 (ring A OCH_3); 55.1 (ring C OCH_3).

(4) 2,3,4-Trimethoxy-1,1′-biphenyl-4′-carboxylic acid (TBC). 2,3,4-Trimethoxy-4′-carbomethoxy-1,1′-biphenyl was refluxed in ethanolic sodium hydroxide (2:1 EtOH to 10% NaOH(aq)) for 3 h. The reaction mixture was evaporated to a solid residue at reduced pressure. The residue was taken up in 0.2 N HCl, and the product was precipitated by adding concentrated HCl. It was extracted by shaking with $CaCl_2$. After the usual workup of the organic solution, the product was obtained in 87% yield. It was recrystallized by dissolving it in a minimal amount of boiling CH_2Cl_2 and then adding an equal volume of medium petroleum ether. Mp: 194.0–194.5 °C. MS: MW 288 (theory 288). UV: λ_{max} 282 nm at pH 1.2, 270 nm at pH 6.0, 272 nm at pH 12. IR (CCl_4): 1682 (C=O); 1606 (aromatic); 1412 (aromatic); 1280 (C–O); 1080 (aromatic) cm^{-1}. ^{1}H NMR (DMSO): δ 7.97 [d, J = 8.4 Hz, H-C(3′), H-C(5′)]; 7.57 [d, J = 8.4 Hz, H-C(2′), H-C(6′)]; 7.08 [d, J = 8.7 Hz, H-C(6)]; 6.89 [d, J = 8.7 Hz, H-C(5)]; 3.82 (s, ring A m-OCH_3); 3.78 (s, ring A p-OCH_3); 3.60 (s, ring A o-OCH_3). ^{13}C NMR (DMSO): δ 167.3 (C=O); 153.6 (C-2); 150.9 (C-4); 142.4 (C-1′); 142.2 (C-3); 129.3 (C-3′, C-5′); 129.0 (C-2′, C-6′); 128.9 (C-4′); 126.6 (C-1); 124.7 (C-6); 108.4 (C-5); 60.9 (ring A OCH_3); 60.5 (ring A OCH_3); 56.0 (ring A OCH_3).

(5) 2,3,4-Trimethoxy-1,1′-biphenyl-4′-ol (TBO) was prepared by the same procedure as the acid from 2,3,4-trimethoxy-1,1′-biphenyl-4′-acetate. The reaction mixture was only warmed, not boiled. Yield: 94%. Mp: 170.0–171.0 °C. MS: MW 260 (theory 260). UV: λ_{max} 274 nm at pH 12, 257 nm in 15% CH_3OH/H_2O. IR (CCl_4): 1490 (aromatic); 1268 (OH); 1218 (OH); 1109 (aromatic); 1086 (aromatic) cm^{-1}. ^{1}H NMR (DMSO): δ 9.49 (s, OH); 7.26 [d, J = 8.4 Hz, H-C(2′), H-C(6′)]; 6.95 [d, J = 8.7 Hz, H-C(6)]; 6.81 [d, J = 8.7 Hz, H-C(5), H-C(3′), H-C(5′)] 3.79 (s, ring A m-OCH_3); 3.76 (s, ring A p-OCH_3); 3.55 (s, ring A o-OCH_3). ^{13}C NMR (DMSO): δ 156.4 (C-4′); 152.4 (C-2); 150.8 (C-4);

[1] Abbreviations: TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; TMB, 2,3,4,4′-tetramethoxy-1,1′-biphenyl; TBO, 2,3,4-trimethoxy-1,1′-biphenyl-4′-ol; TBC, 2,3,4-trimethoxy-1,1′-biphenyl-4′-carboxylic acid; TBA, 2,3,4-trimethoxy-1,1′-biphenyl-4′-acetate; HMB, 2,2′,3,3′,4,4′-hexamethoxy-1,1′-biphenyl; TMB′, 2,4,4′,6-tetramethoxy-1,1′-biphenyl; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; NAM, *N*-acetylmescaline; TME, tropolone methyl ether; GTP, guanosine 5′-triphosphate; PG buffer, 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0, buffer.

142.2 (C-3); 129.9 (C-2′, C-6′); 128.6 (C-1′); 127.9 (C-1); 124.3 (C-6); 115.1 (C-3′, C-5′); 108.2 (C-5); 60.5 (2 ring A OCH_3); 55.9 (ring A OCH_3).

Other Procedures. Ligand-induced GTPase activity was assayed by the release of [^{32}P]phosphate in 10 mM sodium phosphate–1 mM $MgCl_2$ buffer, pH 7.0, containing 0.1 mM [γ-^{32}P]GTP, at 37 °C (Andreu & Timasheff, 1981). Tubulin purified by means of the modified Weisenberg procedure was subjected to Sephacryl S-300 chromatography to eliminate the ligand-independent GTPase activity. The protein samples (1 mg/mL) and blanks were preincubated in the experimental buffer containing ca. 5×10^6 dpm [γ-^{32}P]GTP and a fixed final substrate concentration of 0.1 mM GTP, in a final volume of 0.2 mL. The samples were then incubated 5 min at 25 °C in order to allow isotopic equilibration of exchangeable sites (a control measurement of the small amount of GTP hydrolyzed during this step was made and this value was subtracted from the final amount of hydrolyzed substrate). The enzyme reaction was started by adding the ligand and placing the tubes in a 37 ± 0.5 °C water bath and stopped after 30 min by addition of 0.5 mL of ice-cold water. Then, 0.25 mL of 2.5% ammonium molybdate in 5 N H_2SO_4 was added. The phosphomolybdate complex formed was extracted into 1 mL of an isobutyl alcohol–cyclohexane mixture (1:1 v/v). The organic phase aliquots were counted in a LKB 1219 Rackbeta liquid scintillation counter.

Binding measurements of analogues were done as described in the preceding paper. The binding of colchicine and MTC and their inhibition by analogues were measured as in the preceding paper. The in vitro assembly of microtubules was performed in 10 mM sodium phosphate, 0.1 mM GTP, 0.5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 6 mM $MgCl_2$, and 3.4 M glycerol, pH 6.5, buffer (assembly buffer) at 37 °C in a thermostated cuvette, and the mass of polymer formed was followed turbidimetrically at 350 nm (Lee & Timasheff, 1977) with a Varian 635 spectrophotometer. The self-assembly of tubulin into abnormal polymers in the presence of the ligands in PG and 16 mM $MgCl_2$ buffer at pH 7.0 and 37 or 42 °C was followed turbidimetrically at 350 nm. The polymers formed were fixed with 0.5% glutaraldehyde, adsorbed to formvar carbon-coated grids, negatively stained with 2% uranyl acetate, and examined under a Philips EM 300 electron microscope.

Results

Inhibition of Colchicine and MTC Binding to Tubulin by the Ligands. The specificity of interaction of the ligands TBO, TBC, TBA, HMB, and TMB′ was probed by determining their inhibition of the bindings to tubulin of colchicine and of MTC, a well-characterized equilibrium probe for the colchicine-binding site (Andreu et al., 1984). These experiments were made relative to TMB (characterized in the preceding paper). The results are shown in Figure 1. The most potent inhibitor was TMB, followed by TBO; HMB displayed a weaker, but significant, inhibition; there was a weak inhibition with TBA and TMB′, while TBC gave no inhibition even at a level of 400 μM. The inhibition of the binding of MTC to tubulin followed a pattern very similar to that found for the inhibition of colchicine binding, as shown in Figure 2. Again, the phenol (TBO) and hexamethoxy (HMB) analogues gave reasonable inhibition, while the others either did not inhibit at all or displayed only a marginal effect. Use of higher concentrations of analogues was not possible due to their low solubility.

Binding Equilibrium. The inhibition of MTC binding by the different ligands (Figure 2B) was employed to estimate the magnitude of apparent binding constants by the same

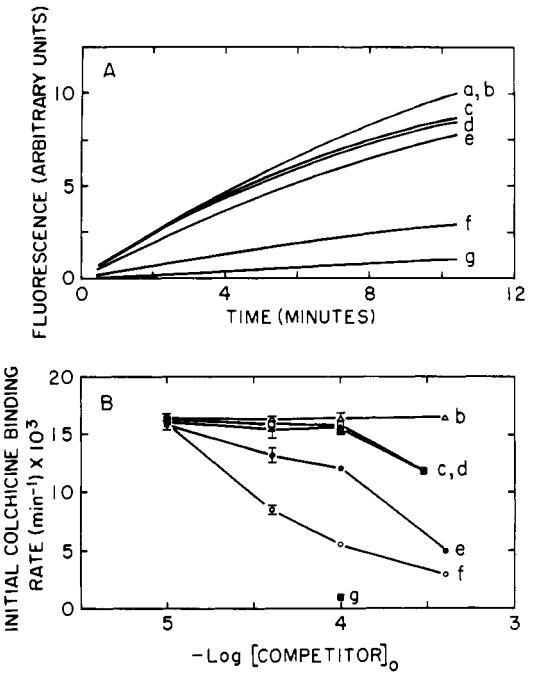


FIGURE 1: Inhibition of colchicine binding to tubulin by biphenyl analogues. Panel A: time course of binding of 10^{-5} M colchicine to 10^{-5} M tubulin at 25 °C, monitored by ligand fluorescence (excitation at 365 nm, emission at 430 nm). Key: (a) no competitor; (b–g) 10^{-4} M biphenyl analogue, (b) TBC, (c) TBA, (d) TMB′, (e) HMB, (f) TBO, and (g) TMB. Panel B: inhibition of the initial rate of colchicine binding by biphenyl analogues. Colchicine and the competitor were added to the protein solution as small volumes of concentrated solutions and mixed simultaneously to start the reaction. Fluorescence units were converted into concentration units by employing appropriate standards of purified tubulin–colchicine complex, and the results are expressed in moles of colchicine bound per moles of tubulin per minute. The analogue for each time course is as in panel A.

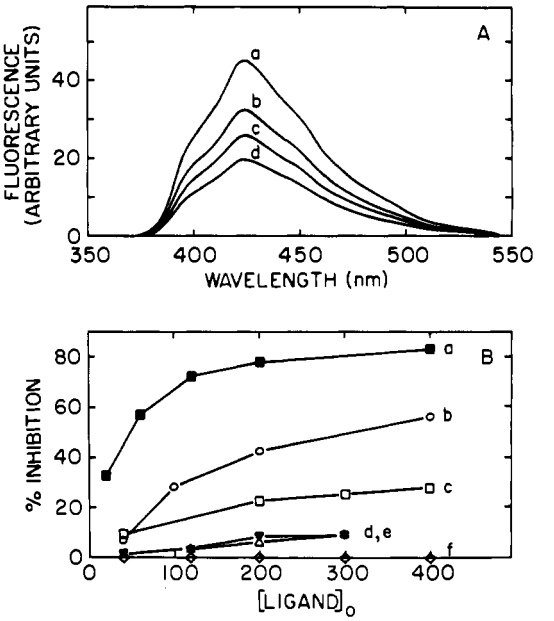


FIGURE 2: Panel A: inhibition of MTC binding to tubulin by TBO. Fluoresence emission spectra (excitation at 350 nm) of 10^{-5} M MTC bound to 10^{-5} M tubulin at 25 °C in the absence (a) or in the presence of (b) 10^{-4} M, (c) 2.0×10^{-4} M, or (d) 4.0×10^{-4} M TBO. Panel B: percentage of inhibition of MTC binding to tubulin by (a) TMB, (b) TBO, (c) HMB, (d) TBA, (e) TMB′, and (f) TBC as a function of ligand concentration. The emission wavelength was 423 nm.

fitting procedure as in the preceding paper. The values obtained for TMB, TBO, and HMB were 8.2×10^4, 1.8×10^4, and ca. 1.3×10^3 M^{-1}, respectively. The other ligands were

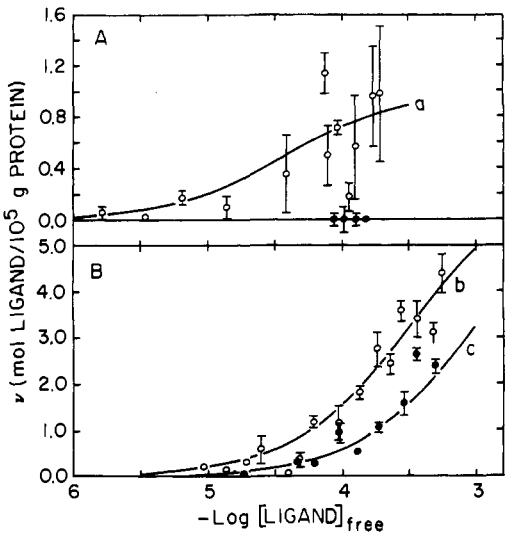


FIGURE 3: Binding isotherms of TMB (panel A) and TBO (panel B) to tubulin (open symbols) and to the tubulin–colchicine complex (filled symbols). The measurements were made on (5–8) × 10^{-6} M protein in PG buffer, pH 2.0, 25 °C, by employing the centrifugation method (Materials and Methods). The large errors are the result of partial ligand adsorption to the centrifuge tubes.

not significantly inhibitory. The binding of the phenol analogue (TBO) to tubulin was compared semiquantitatively to that of TMB by centrifugation of the protein, followed by the appropriate spectrophotometric measurement of free ligand in the supernatant. The results are presented in Figure 3. As shown in the upper panel, TMB binds to ca. one site on tubulin, while its binding is totally blocked by formation of the tubulin–colchicine complex. The phenol analogue, on the other hand, binds to a larger number of sites (>6) in a nonsaturable manner. This binding is diminished by approximately one molecule of TBO when tubulin is changed into the tubulin–colchicine complex, over the concentration range available to measurement. Therefore, TBO has the ability to bind to the colchicine site on tubulin, but it can also bind to a number of nonspecific sites. Saturation could not be reached at TBO concentrations up to 0.6 mM, suggesting multiple binding, similar to that found with bisANS and daunomycin (Ward and Timasheff, manuscript in preparation).

Conformational Effects of the Ligands. The binding of colchicine to tubulin has as consequences (i) the induction of a small change in the CD spectrum in the 217–220-nm region, (ii) the appearance of GTPase activity, (iii) the inhibition of microtubule formation, and (iv) the tubulin polymerization into structures that geometrically differ from microtubules, but have the same thermodynamic assembly behavior (Andreu & Timasheff, 1982b; Andreu et al., 1983). On binding to tubulin, TKB induced the CD perturbation (Medrano et al., 1991), while neither TMB nor the same concentration of TBO (2 × 10^{-5} M) had any effect.

The results of measurements of the GTPase activity induced in tubulin by the various ligands are presented in Table I. All binding ligands (TKB, TMB, TBO, and HMB) induced a GTPase activity in tubulin. The maximal rates obtained in PG buffer and 1 mM $MgCl_2$, pH 7.0, containing 1% dimethyl sulfoxide were similar to those reported previously for ALLO and TCB (Medrano et al., 1989). All were somewhat lower than colchicine.

Effects of Ligands on the in Vitro Microtubule Assembly. The effects of the four binding analogues (TKB, TMB, TBO, and HMB) on the in vitro microtubule assembly are shown in Figure 4. All four ligands inhibited the polymerization.

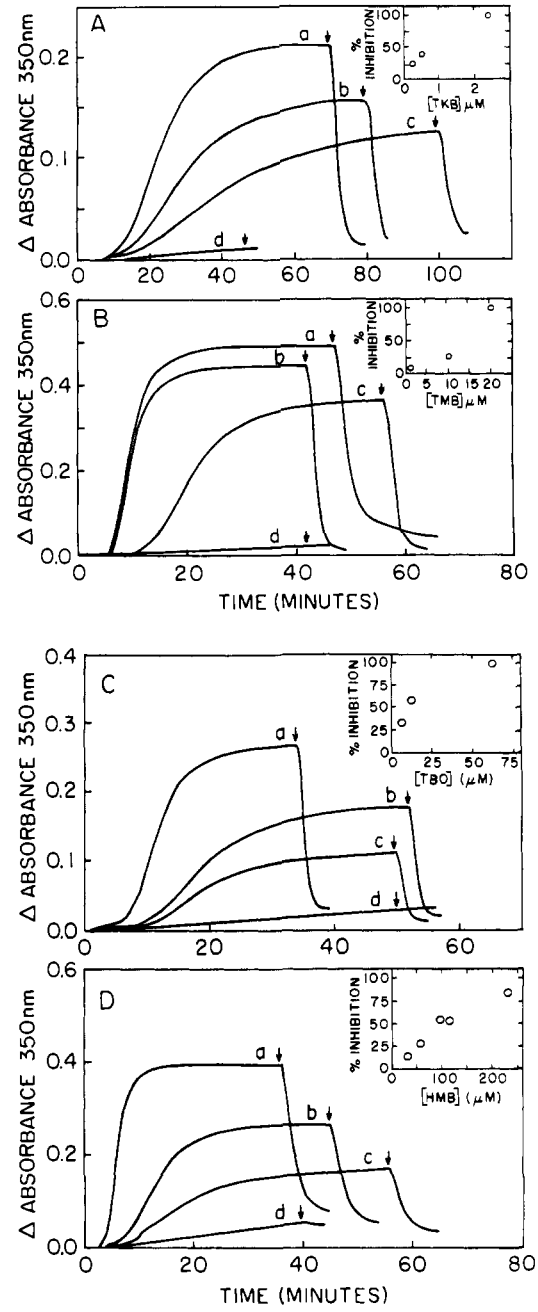


FIGURE 4: Effects of biphenyl ligands on the turbidity time course of the in vitro microtubule assembly. The reaction was started by warming the solution from 10 to 37 °C; the arrows indicate cooling of the samples to 10 °C. Panel A: (a) 1 × 10^{-5} M tubulin; (b–d) aliquots of the same solution with 2.7 × 10^{-7}, 5.3 × 10^{-7}, and 2.38 × 10^{-6} M TKB. Panel B: (a) 1.48 × 10^{-5} M tubulin; (b–d) aliquots of the same solution with 1.04 × 10^{-6}, 1.01 × 10^{-5}, and 2.01 × 10^{-5} M TMB. Panel C: (a) 1.15 × 10^{-5} M tubulin; (b–d) aliquots of the same solution with 6.30 × 10^{-6}, 1.25 × 10^{-5} and 6.25 × 10^{-5} M TBO. Panel D: (a) 1.43 × 10^{-5} M tubulin; (b–d) aliquots of the same solution with 5.57 × 10^{-5}, 1.15 × 10^{-4}, and 2.30 × 10^{-4} M HMB. The insets show the percentage of turbidity inhibition as a function of ligand concentration.

As shown in Figure 4A, the turbidity generated by the self-assembly of 1.35 × 10^{-5} M pure tubulin was reduced by 50% by 6 × 10^{-7} M TKB, indicating a substoichiometric mode of inhibition. TMB also inhibited microtubule assembly (Figure 4B). The concentration of TMB necessary to reduce by 50% the turbidity generated by the self-assembly of 1.48 × 10^{-5} M tubulin was 1.3 × 10^{-5} M. For TBO and HMB, the turbidity generated by the self-assembly of 1.15 × 10^{-5} M pure tubulin was reduced by 50% by 8.0 × 10^{-6} M TBO and that

Table I: Ligand-Induced GTPase Tubulin Activity

ligand	concn (μM)	activity[a]
colchicine		100
allocolchicine	200	62
TCB	200	48
TKB	200	38
TMB	200	29
TBO	500	42
HMB	500[b]	10
TBA		nd
TMB′		nd
TBC	500	4
podophyllotoxin	200	2

[a] GTP hydrolysis rate relative to the tubulin–colchicine complex. [b] This was practically the solubility limit of this ligand, at which only a partial inhibition of colchicine and MTC binding was observed (Figures 1 and 2). At this concentration, it is only ca. 25% bound. Therefore, the small GTPase activity detected may be regarded as significant.

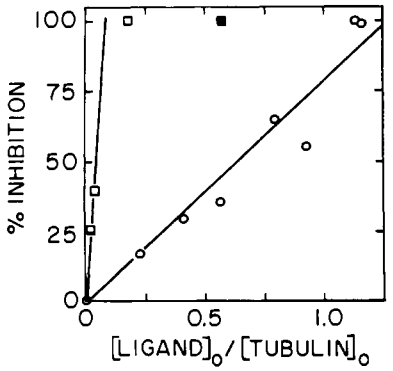


FIGURE 5: Percentage of inhibition of the plateau absorbance values of the polymerization of tubulin by TMB (○) or by TKB (□) in assembly buffer at 37 °C, as a function of the ratio of total ligand concentration to total protein concentration. The protein concentration was 8.8 × 10^{-5} M in the TMB experiments and at the highest point with TKB (■); it was 1 × 10^{-5} M in the first three TKB points (□).

generated by the self-assembly of 1.43 × 10^{-5} M pure tubulin was reduced by 50% by 1.0 × 10^{-4} M HMB. Electron microscopic examination showed that, in the presence of all four drugs, the morphology of the polyermization product under inhibition conditions was indistinguishable from that of microtubule controls assembled without drugs (not shown). The ligands TBA or TMB′, at a concentration of 2.4 × 10^{-4} M, or TBC, at 2.2 × 10^{-4} M, had no effect on the assembly of 1.1 × 10^{-5} M tubulin. These results indicate that the inhibition of microtubule assembly by TKB is substoichiometric, while that by the other inhibiting ligands is stoichiometric. To ascertain further the character of the inhibition by TMB, inhibition measurements were performed at a higher concentration of tubulin (8.85 × 10^{-5} M). As shown in Figure 5, the extent of inhibition by TMB increased linearly with the mole ratio of total ligand to total protein in the solution, 50% inhibition occurring at a mole ratio of 0.6 mol of analogue/mol of tubulin, while for TKB this occurred at a mole ratio of 0.04. It is clear, therefore, that, in contrast to TKB, TMB inhibits microtubule assembly in a close to stoichiometric manner.

Induction of Abnormal Polymer Formation. An excess of TKB (7.5 × 10^{-5} M) induced an increase in turbidity in PG buffer and 16 mM $MgCl_2$, pH 7.0, when the solution was heated to 37 °C, as shown by the solid lines in Figure 6A, but no microtubules were seen under the electron microscope. This polymerization was reversed by cooling to 10 °C. The process was characterized by a lag time and a critical concentration, C_r = 1.0 ± 0.1 mg/mL tubulin, which is characteristic of nucleated cooperative self-assembly (Oosawa & Asakura,

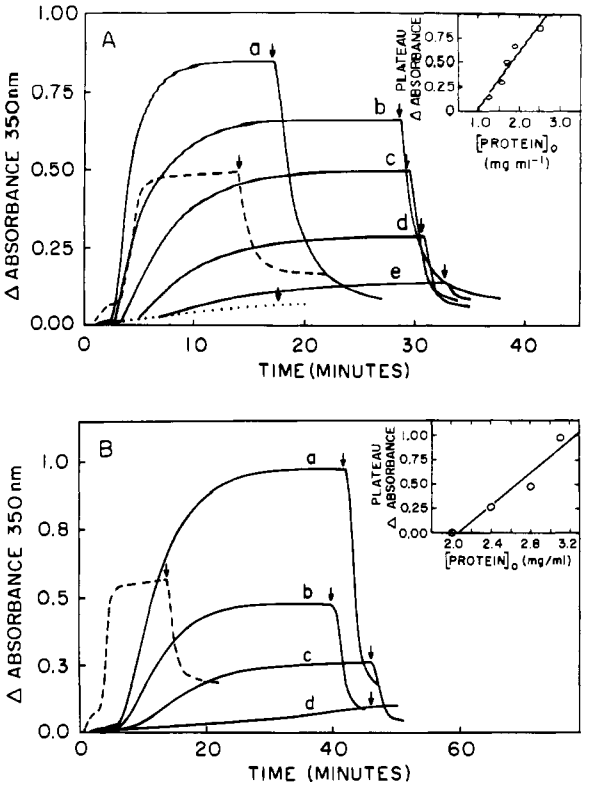


FIGURE 6: Turbidity time course of the abnormal polymerization of tubulin induced by the biphenyl ligands in PG and 16 mM $MgCl_2$ buffer, pH 7.0. The arrows indicate cooling of the samples to 10 °C. Panel A: (solid lines) polymerization of tubulin induced by 7.5 × 10^{-5} M TKB, when heated from 10 to 37 °C [concentrations of tubulin were (a–e) 2.52, 1.89, 1.70, 1.58, and 1.26 mg mL^{-1}; inset shows the plateau absorbance values of the abnormal polymerization induced by TKB as a function of total protein concentration]: (dotted line) polymerization of 3.15 mg mL^{-1} tubulin induced by 7.5 × 10^{-5} M TMB, when heated from 10 to 37 °C; (dashed line) polymerization of 8.1 mg mL^{-1} tubulin induced by 3.75 × 10^{-4} M TMB, when heated from 10 to 42 °C. Panel B: (solid lines) polymerization of tubulin induced by 7.5 × 10^{-5} M TBO, when heated from 10 to 37 °C [concentrations of tubulin were (a–d) 3.1, 2.8, 2.4, and 2.0 mg mL^{-1}; inset shows the plateau absorbance values of the abnormal polymerization induced by TBO as a function of total protein concentration]; (dashed line) polymerization of 8.1 mg mL^{-1} tubulin induced by 5.0 × 10^{-4} M HMB, when heated from 10 to 42 °C.

1975). The formation of anomalous tubulin polymers with a critical concentration of ca. 1 mg/mL by stoichiometric binding of the drug to α–β tubulin is characteristic of colchicine (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983), MTC (Andreu et al., 1984), allocolchicine, and TCB (Medrano et al., 1989). An excess of TMB [(7.5–9.5) × 10^{-5} M] over tubulin [(3.15–6.0) × 10^{-5} M] did not induce any reversible increase in turbidity, apart from nonspecific cold-irreversible aggregation, in PG buffer and 16 mM $MgCl_2$, pH 7.0, when the solution was heated to 37 °C, as shown by the dotted line in Figure 6A. The phenol analogue of TMB, TBO, however did induce the cold-reversible increase in turbidity when heated to 37 °C in the same medium, as shown in Figure 6B. For TBO, the critical concentration was higher than that for TKB, C_r = 2.1 ± 0.1 mg/mL tubulin, and the process was characterized by a lag time somewhat longer than that for TKB. HMB, on the other hand, did not induce any increase in turbidity on heating to 37 °C. The cooperative cold-reversible increase in turbidity could be induced by both TMB and HMB if the temperature was raised to 42 °C or above, as shown by the dashed lines in Figure 6A,B. This required, however, both a high concentration of protein (8.1 mg/mL) and a large excess of ligand (3.75 × 10^{-4}

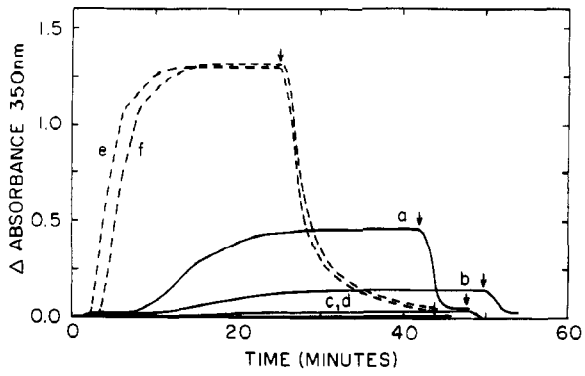


FIGURE 7: Inhibition by TMB of the abnormal polymerization of tubulin induced by TKB (solid lines) and of the tubulin–colchicine complex (dashed lines) in PG–16 mM $MgCl_2$ buffer, pH 7.0. The reaction was started by warming the solution from 10 to 37 °C; the arrows indicate cooling of the samples to 10 °C. Key: (solid lines) polymerization of 2.95×10^{-5} M tubulin induced by 7.5×10^{-5} M TKB in the absence (a) or in the presence of (b–d) 1.25×10^{-5}, 3.75×10^{-5}, and 6.25×10^{-5} M TMB; (dashed lines) polymerization of 3.3×10^{-5} M tubulin–colchicine complex in the absence (e) or in the presence (f) of 6.25×10^{-5} M TMB.

M TMB and 5×10^{-4} M HMB). None of the polymerization products under these conditions had the morphology of microtubules. These results are consistent with microcalorimetric and equilibrium heating and cooling turbidity observations that, in the presence of TKB or colchicine, a cooperative endothermic phenomenon takes place at 35 °C, while with TMB this occurs only at 41 °C (Hinz and Timasheff, unpublished experiments). The incomplete reversibility by cooling can be attributed to protein destabilization at 42 °C.

The polymerization of tubulin at 37 °C in the presence of TKB, and lack of polymerization in the presence of TMB at 37 °C, but not at 42 °C, was used to test directly the competitive binding of TKB and TMB to the same site on tubulin. As shown in Figure 7, TMB abolished competitively the TKB-induced abnormal polymerization of tubulin when heated to 37 °C. Under identical conditions, as excess of TMB had no effect on the abnormal polymerization of the stable tubulin–colchicine complex. These observations are consistent with the notion that both TKB and TMB bind in rapid equilibrium to the colchicine site of tubulin. In identical control experiments, replacement of TMB by MTC, a colchicine site ligand known to induce the abnormal polymerization of tubulin (Andreu et al., 1984), had no significant effect on the development of turbidity in the presence of TKB. Furthermore, 8.5×10^{-5} M TKB was able to induce the abnormal polymerization of 3.2×10^{-5} M tubulin at 42 °C just as TMB did. At this temperature, addition of 1.3×10^{-4} M TMB to the tubulin–TKB mixture had no inhibitory effect on the abnormal polymerization. This is in contrast to the results at 37 °C, where TMB does not support polymerization, and fully consistent with the above interpretation that TKB and TMB bind competitively to the colchicine site on tubulin.

Discussion

Tolerance of Ring C′ Substituents. The results of this and the preceding paper clearly point to the roles of the carbonyl and methoxy oxygens in determining the mode of binding of ring C′ (or C) into the tropolone-binding subsite of the colchicine-binding site of tubulin. The two modes of binding can be classified into the TKB type and the TMB type.

Figure 8 shows the structures of TMB, TKB, TCB, and TBA superimposed onto the crystal structure of colchicine (Lessinger & Margulis, 1978). The modeled structures of TMB and TKB are closely related to the crystal structures of these compounds if in TKB the 4′-substituent is rotated by ca. 180° (M. Rossi, unpublished). The trimethoxyphenyl rings A have been made to coincide for the purpose of the comparison. However, since the binding of the colchicine ring A

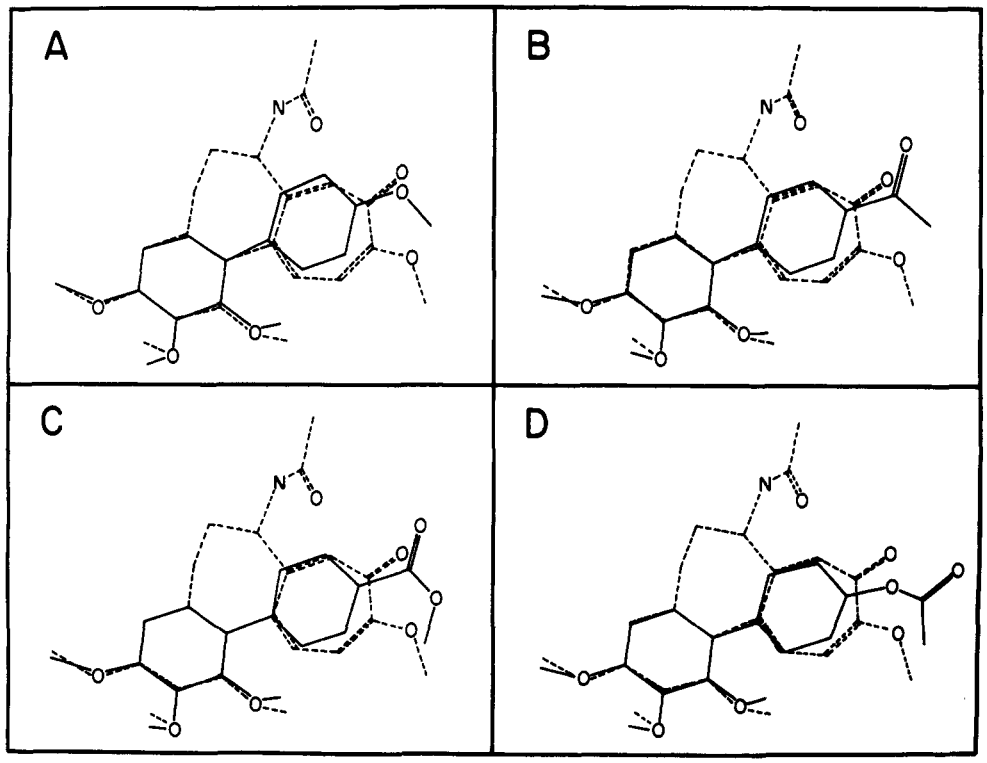


FIGURE 8: Models of TMB (A), TKB (B), TCB (C), and TBA (D) with their trimethoxybenzene rings superimposed onto that of colchicine. The biaryl and methoxy torsion angles were adjusted to the crystallographic colchicine values [colchicine molecule a, Lessinger and Margulis (1978)] and displayed with a personal computer program. The 4′-substituents in TKB, TCB, and TBA have torsion angles arbitrarily chosen to make their oxygens close to the position of the colchicine oxygens.

to its protein subsite appears to be predominantly hydrophobic (Andreu & Timasheff, 1982a,b), some tolerance might be expected, and its positioning into the binding site may not be necessarily coincident. In this comparison the 4′-methoxy oxygen of TMB (Figure 8A) lies at approximately 2.0 Å from the methoxy oxygen of colchicine and 0.6 Å from the carbonyl oxygen. This suggests that TMB might enter into the same hydrogen-bonded interactions as the carbonyl oxygen of colchicine, albeit more weakly. The 4′-carbonyl oxygen of TKB can be made to lie at ca. 1.0 Å from either the carbonyl or the methoxy oxygen of colchicine (Figure 8B) by rotation of the 4′-substituent, which would suggest that TKB can enter into hydrogen-bonded interactions similar to those of the colchicine oxygens. The 4′-carbonyl and 4′-methoxy oxygens of TCB can lie close to the carbonyl and methoxy oxygens of colchicine (ca. 0.6 and 1.0 Å, respectively, see Figure 8C). Therefore, for TCB, hydrogen-bonded interactions similar to those of colchicine might be envisaged. Analogous reasoning to the above could be applied if, instead of the crystal structure of colchicine, the quasiplanar conformer (Detrich et al., 1981) was employed.

Further modification of the 4′-substituent reduced the binding and, as a corollary, the ability to inhibit microtubule assembly. Inversion of the ester to TBA (Figure 8D) greatly weakened the interaction. This must reflect the spatial constraints on the binding, as the carbonyl oxygen is moved to a position ca. 2.6 Å distant from the equivalent oxygen of colchicine and, therefore, is unfavorable for the TKB type of interaction. The inability of the ester oxygen of TBA to substitute for the methoxy oxygen of TMB, even though the two are in similar positions, is probably due to steric blocking by the rest of the TBA substitutent (see Figure 8D). Conversion of the TCB ester ($COOCH_3$) to its acid (COOH) abolished all binding ability. At the conditions used, pH 7.0, this group must be ionized. It is possible, therefore, that in the carboxylate form it cannot penetrate the site due either to electrostatic repulsion or to the large amount of free energy needed ($\sim$4 kcal mol^{-1}) to enter into a nonpolar environment. Conversion of the TMB methoxy to its phenol (TBO) had two effects: (i) it weakened the binding; (ii) it strengthened the induction of abnormal polymerization. Since the phenolic hydroxyl is protonated at pH 7.0, this result is consistent with a modulation of the interactions by hydrogen bonds. The hexamethoxy compound, which is a dimer of ring A, displayed binding greatly reduced from TMB, from which it differs by the introduction of methoxy groups into positions 2′ and 3′ of ring C, suggesting steric interference.

Consequences of the Binding of the Various Ligands. First, all the biphenyl ligands that bound to tubulin in the colchicine site induced the GTPase activity. This is the most nondiscriminatory property of the complex. In fact, GTPase activity is known to be induced in tubulin by other drugs that bind at sites other than the colchicine-binding site (David-Pfeuty et al., 1979). In the case of the colchicine analogues, binding to both the ring A and ring C subsites seems to be required, since podophyllotoxin, which binds to the ring A locus only, does not induce this activity.

The difference between a carbonyl and methoxy group in position 4′ in its effects on tubulin is brought out strikingly in the perturbation of the far-UV circular dichroism spectrum and inhibition of microtubule formation. All carbonyl-containing ligands examined (colchicine, allocolchicine, MTC, TKB, and tropolone methyl ether) induced a weak perturbation in tubulin CD at 217–220 nm. The same ligands inhibited microtubule assembly substoichiometrically. On the other hand, TMB, which has a methoxy group in that position, and apparently TBO, did not perturb the tubulin CD in the far UV region, and both inhibited microtubule assembly stoichiometrically. For TCB, the ester, the situation is intermediate. It induced a weak perturbation of the CD but inhibited microtubule assembly stoichiometrically. There seems, therefore, to be a relation between the effect on the asymmetric environment of a chromophoric locus on tubulin and the ability to inhibit microtubule formation by a substoichiometric mechanism, i.e., by blocking the growth end of a microtubule (Margolis & Wilson, 1977).

Finally, the induction of the abnormal polymerization of tubulin by colchicine analogues again seems to be related to the nature of the hydrogen-bond acceptor and the ability to anchor the ligand in proper orientation in the ring C binding site in tubulin. All the carbonyl-containing compounds induced this self-assembly at 37 °C, as did TCB. The phenolic compound also induced polymerization at 37 °C, but with a higher critical concentration. The methoxy analogues, TMB and HMB, required heating to a higher temperature, which indicates a weaker reaction. While lack of knowledge of the three-dimensional structure of tubulin precludes a detailed analysis of its colchicine- and analogue-induced assembly, it is reasonable to assume that the effect is on lateral bond formation, since the longitudinal bonds along protofilaments appear to be chemically invariant in the different types of assemblies undergone by tubulin. The lateral bonds, however, are highly susceptible to spatial perturbations in the mutual alignment of the protein subunits (Melki et al., 1989), and the various ligands may induce slightly different conformational states in tubulin.

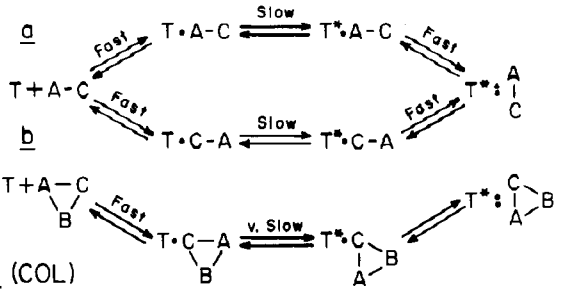
Differences between the Ring A and the Ring C Binding Subsites. Role of Ring B. The results of these studies are fully consistent with the two-subsite model of colchicine binding to tubulin, one subsite specific for ring A and the other for ring C, both sites being present on the tubulin molecule and available for the independent binding of ring A and ring C types of structures (Andreu & Timasheff, 1982a,b). Recently, the validity of this model has been questioned, and an alternate model based on results obtained with the drug combretastatin A4 has been proposed (Lin et al., 1989). This consists of two identical subsites both specific for ring A that can accommodate with some difficulty a tropolone ring (ring C). These two subsites would be in homologous locations on the α- and β-tubulin polypeptide chains. Therefore, the complete colchicine site would span the two subunits, which would be related either by a plane of symmetry or by a translation [see Figure 5 in Lin et al. (1989)]. There are several structural and biochemical reasons why this model is difficult to accept. First, a head to head symmetric arrangement of the α and β subunits within each heterodimer along the protofilament in the microtubule would violate the accepted basic 4-nm repeat along the protofilament (evidenced by electron microscopy and X-ray fiber diffraction of microtubules) that corresponds to the tubulin monomer (Amos, 1979; Mandelkow et al., 1977). Also, a head to head disposition either would require a complementary subunit-specific tail to tail interaction (α with β, but not α with α) or would render the microtubule growth and dissociation ends chemically identical, which is contrary to the current understanding of the polar microtubule polymerization (Margolis & Wilson, 1981; Mitchinson & Kirchner, 1986). Second, the α and β subunits being in polar alternate arrangement within the heterodimer and the protofilament, the chemically homologous sequences constituting the two similar ligand-binding subsites

would be expected to be located in structurally homologous positions and, therefore, displaced by 4 nm from each other, a distance spanned by neither the colchicine nor the combretastatin molecules. There are also a number of specific consequences that this model would entail, none of which are observed: (i) If both subsites were tailored to a benzene ring, allocolchicine should bind more strongly than colchicine, and TCB more strongly than MTC; both are untrue. (ii) *N*-Acetylmescaline (NAM) and tropolone methyl ether (TME) should show binding stoichiometries of 2; in fact, the binding stoichiometry of TME is 1. (iii) The binding of NAM and TME should show competition; in fact, they are independent of each other. (iv) The enthalpies of binding of NAM and TME should be identical (same mechanism of binding); in fact, TME binding is enthalpy driven, while NAM binding is entropy driven. (v) All analogues should induce the change in CD at 220 nm; yet, TMB, in which ring C is most similar to ring A, does not induce this change. (vi) If the specificity of both sites were for ring A, then the hexamethoxy compound (HMB) should be the strongest binder as it is a dimer of ring A; in fact, its binding is weak.

The model based on the two chemically different subsites, one hydrophobic (ring A) and the other stacking (ring C), on the other hand, can account for all the results described on the binding of colchicine, MTC, allocolchicine, and the biphenyl analogues. As proposed before (Medrano et al., 1989), the contribution of ring B to the thermodynamics is strictly through the elimination of the freedom of rotation about the A–C bond in colchicine. Kinetically, ring B most probably constitutes a transient steric impediment that must be overcome in the approach of ring A to its binding subsite. This large activation energy barrier becomes superimposed on that of the protein conformational change. Comparison between the E_a values measured for colchicine and MTC suggests that the presence of ring B essentially doubles this quantity. This analysis is consistent with studies described on the effect of ring B on colchicine binding to tubulin (Bhattacharyya et al., 1986; Banerjee et al., 1987). In both studies, the effect of varying the substitutent in ring B on the rate of binding has been examined. It seems clear that an increase in the bulk of the ring B side chain increases the energy barrier to binding. The same appears to be true for the reverse reaction, as the rate of dissociation of the des(acetylamido)colchicine–tubulin complex is greatly enhanced relative to that of colchicine (Banerjee et al., 1987), which should reflect the decrease in the bulkiness of the ring B moiety of the molecule to which the side chain makes a significant contribution (Rossi et al., 1984). It is, of course, not excluded that introduction of polar or reactive groups into ring B or its side chain might generate new productive contacts with loci on the protein not normally involved in the binding of colchicine.

Thermodynamics and Kinetics of Binding to the Colchicine Site. The two-subsite model of binding of colchicine to tubulin (Andreu & Timasheff, 1982a) is essentially a thermodynamic model, and, as such, it does not imply any particular kinetic pathway. The characteristics of the tubulin–ligand interactions, however, and the similarity of the thermodynamic parameters of binding of TME to those of the first step of the kinetic scheme proposed for the binding of colchicine to tubulin (Garland, 1978; Lambeir & Engelborghs, 1981) led to the hypothesis that colchicine ring C should bind first, followed by a slow conformational change allowing the binding of ring A (Andreu & Timasheff, 1982a,b). The binding of colchicine, therefore, can be described by the kinetic pathway marked b′ in Scheme I. Engelborghs and Fitzgerald (1987) pointed out

Scheme I



that an identical model could not hold for MTC binding to tubulin, since the thermodynamic parameters of the first step of the two-step binding processes are drastically different for colchicine and MTC. This is fully correct. Nevertheless, the identical bidentate sequential binding process can account for MTC binding if the order of events can be reversed, i.e., if in MTC ring A can bind first. Can this be justified? Examination of the three-dimensional structure of colchicine shows that, for this ligand, initial binding of ring A should be a rare event. The activity of ring A of colchicine appears to involve the interaction of its *o*-methoxy group with the protein (Brossi et al., 1988). In colchicine, this group is located on the same side of the plane of the benzene ring as the bulky structure of ring B (Lessinger & Margulis, 1978; Rossi et al., 1984) (see Figure 8). This should render the fixing of ring A into its subsite difficult because of steric hindrance, i.e., of an activation energy barrier (Margulis, 1975) that would need to be overcome. Ring C is not hindered in such a manner, as both oxygens point away from the bulk of ring B. As a result, it interacts first. In MTC, ring A is free to interact with its binding site.

These considerations suggest a simple two-subsite model that can account for the binding of both colchicine and MTC. It is based on all the currently available thermodynamic parameters of these processes and is consistent with them. This model is depicted in Scheme I. It consists of two alternate pathways, a and b, with the end tubulin–ligand complex identical in the two cases. In both, a protein conformational change, which follows the initial binding, is the rate-determining step. This renders the second binding event not separable in thermodynamic measurements from the conformational change. In the case of pathway a, the conformational change that follows the binding of ring A either precedes or is concerted with the binding of ring C. For purposes of thermodynamic argument we have drawn it as the second step in the sequence of reactions. The two processes can be assessed, then, in terms of three subreactions by a concerted analysis of all the thermodynamic data on colchicine and MTC bindings. Such an analysis requires that all non-reaction-specific entropic terms be properly accounted for. This is accomplished by reducing all the standard entropy changes to unitary entropy, $\Delta S_u^\circ = \Delta S^\circ - \Delta S^\circ_{cratic}$ and by the calculation of the unitary standard free energy change, ΔG_u°. This permits comparison in a rigorously correct way of the strengths of the bindings. The resulting values are listed in Table II. In the case of MTC a further correction is required for the loss of the entropy of rotation about the bond between rings A and C. This has a value between 4 and 10 eu (Glasstone, 1940; Benson, 1976; Orville-Thomas, 1974). The first striking result of this comparison is that the intrinsic strengths of binding of colchicine and MTC are identical within experimental error, as are their standard enthalpy and entropy changes. This fact establishes definitively that ring B makes no thermodynamic contribution to the binding.

Table II: Thermodynamic Parameters of the Binding of Colchicine and Analogues to Tubulin at 37 °C

ligand	$\Delta G°$(exptl) (kcal mol^{-1})	$\Delta G_u°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$(exptl) (cal mol^{-1} K^{-1})	$\Delta S_u°$ (cal mol^{-1} K^{-1})
colchicine	−9.8[a]	−12.3	−5.0[b]	+15.5	+23.5
1st step[c]	−2.8	−5.3	−7.9	−16.5	−8.5
2nd step[d]	−7.0	−7.0	+2.9	+32.0	+32.0
MTC	−7.8[e]	−10.3	−4.6[b]	+10.4	+18.4
MTC (intrinsic)		−12.2	−4.6		+24.4
1st step[f]	−3.4	−5.9	−0.2	+10.3	+18.3
2nd step[d]	−4.4	−4.4	−4.4	+0.1	+0.1
2nd step (intrinsic)		−6.3			+6.1
TME[g]	−3.6	−6.1	−8.3	−15.1	−7.1
NAM[g]	−3.5	−6.0	+3.3[h]	+22.6[h]	+30.6[h]
70% NAM + 30% TME	−3.6	−6.1	−0.2[i]	+11.3[i]	+19.3[i]
30% NAM + 70% TME	−3.6	−6.1	−4.8[i]	−3.8[i]	+4.2[i]
ALLO	−8.0[j]	−10.5	−2.6[b]	+18.6	+26.6

[a] Diaz and Andreu (1991). [b] Menendez et al. (1989). [c] Lambeir and Engelborghs (1981) (see Figure 4 and text). [d] Calculated by difference. [e] Andreu et al. (1984). [f] Engelborghs and Fitzgerald (1987). [g] Andreu and Timasheff (1982a). [h] The NAM $\Delta H°$ and $\Delta S°$ are estimated values, calculated by direct application of the bifunctional ligand model (Andreu & Timasheff, 1982a) to the binding parameters of colchicine and tropolone methyl ether shown in the table; these values are given solely for the purpose of discussion. [i] Values are derived from NAM $\Delta H°$ or $\Delta S°$; therefore, they are tentative. [j] Medrano et al. (1989).

The first step in the binding of colchicine is described by parameters similar to those of TME binding. For MTC, the identification of the first step requires knowledge of the enthalpy change of NAM binding. While at present there is no exact measurement of this parameter, the available data and calculations point to a small positive value (Table II), as well as a positive entropy value. These parameters are qualitatively similar to the parameters of the first step of MTC binding, which suggests the participation of ring A in the initial binding of MTC. A linear combination of the NAM and TME thermodynamic binding parameters given in Table II, however, is consistent with the experimentally determined parameters for the first step of MTC binding. This suggests that in the binding of MTC both kinetic pathways a and b of Scheme I should be open and binding may be initiated by random first contact of either ring A or ring C. This is shown in Figure 9, which presents the kinetic reaction progress for the two pathways, as well as the one experimentally determined by Engelborghs and Fitzgerald (1987). It is clear that the last is represented well by a linear combination of pathways a and b.

What is the nature of the second step of binding? The thermodynamic parameters of this step consist of contributions from both the binding of the second ring and the conformational change that accompanies it (Scheme I). Therefore, $\Delta G°$(second step) = $\Delta G°$(conformational change) + $\Delta G°$(second ring). For colchicine binding it follows that $\Delta G°$(conformational change) = −1.0 kcal mol^{-1}, which corresponds to an equilibrium constant of 5.0. In the case of MTC the directly calculated value of $\Delta G°$(conformational change) is +1.7 kcal mol^{-1}, if the loss of the entropy of rotation about the biaryl bond is not taken into account. Setting the last parameter equal to −6 cal mol^{-1} K^{-1} (Glasstone, 1940; Benson, 1976; Orville-Thomas, 1974) gives a value for $\Delta G°$(conformational change) of −0.2 kcal mol^{-1} (K = 1.4), i.e., a situation essentially similar to that of colchicine. This analysis suggests, therefore, that the rate-determining conformational change in both colchicine and MTC binding is a thermodynamically nearly indifferent reaction that is independent of the order of binding of the two rings (pathways a and b in Scheme I). A rigorous analysis of the enthalpy and entropy changes of the second step of binding to the colchicine site in terms of the contributions of the conformational change and the binding of the second ring would require calorimetric measurements of the enthalpies of binding of NAM and TME to tubulin.

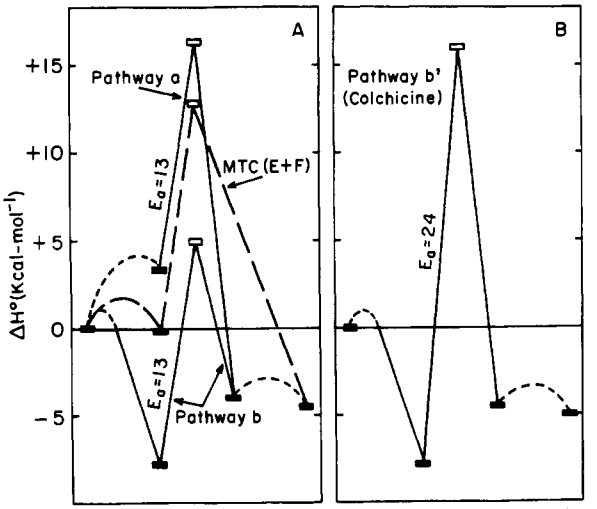


FIGURE 9: Kinetic pathways for the bindings of MTC (A–C) and colchicine to tubulin. The standard enthalpy change values ($\Delta H°$) are taken from Table II. The values of the activation energies (E_a), expressed in kilocalories per mole, are taken from (colchicine) Garland (1978) and Lambeir and Engelborghs (1981); (MTC) Engelborghs and Fitzgerald (1987). Reaction pathways a and b correspond to Scheme I (see text for details). Pathway b′ is for colchicine, for which the second step is very slow due to the steric hindrance caused by ring B. In panel A, the reaction pathway MTC (E + F), drawn as long dashes, is that given by Engelborghs and Fitzgerald (1987) for the fast phase of MTC binding. It is seen to lie between pathways a (binding of ring A first) and b (binding of ring C first). In this presentation the activation energy of the second step in the binding process has been taken as being identical at 13 kcal mol^{-1} for pathways a and b, i.e., the value determined experimentally by Engelborghs and Fitzgerald (1987).

The same mechanism can be extended in principle to the binding of the biphenyl analogues. For allocolchicine (Medrano et al., 1989) the reaction path should be similar to that of colchicine. By using the same mechanism as for colchicine, the standard free energy change for the binding of ring C′ (carbomethoxybenzene) uncoupled from ring A (a situation similar to the case of TME) can be calculated to have a value of ca. −2.3 kcal mol^{-1} i.e., a binding constant of ca. 50 M^{-1}. Therefore, it would be hardly measurable. A detailed analysis of the binding of ALLO and the simple biphenyls TCB, TKB, and TMB will require the determination of the calorimetric enthalpies of binding of the biphenyl compounds, as well as the detailed kinetic analysis of their interactions with tubulin.

The thermodynamic parameters of the individual steps further confirm the nature of the interactions involved. The binding of ring C has the characteristics of aromatic ring-stacking interactions that have typical values of $\Delta H^\circ = -8 \pm 3$ kcal mol^{-1} and $\Delta S_u^\circ = -5 \pm 2$ eu (Engelborghs, 1981). These are essentially the values measured for the first step of colchicine binding. The ring A binding parameters are typical for nonpolar interactions (Tanford, 1980). That the primary stabilizing force in the binding of ring C is stacking has been proposed by Hastie on the basis of spectroscopic data (Hastie, 1989; Hastie & Rava, 1989). The same data can explain the fluorescence generated from the onset of the binding process. Ring A does not fluoresce. Its binding, however, brings ring C into an asymmetric nonpolar environment. Fixation in the proper orientation with respect to the transition dipoles of the tryptophan in the protein and locking into position by formation of the proper hydrogen bonds following the slow conformational change should enhance the fluorescence of this weak fluorophor.

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