Linkages in Tubulin-Colchicine Functions: The Role of the Ring C (C′) Oxygens and Ring B in the Controls†

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ABSTRACT: Linkages between structural components of colchicine (COL) and its biphenyl analogues (allocolchicine, ALLO, and its analogues) in the binding to tubulin and its functional consequences were scrutinized. Three ring ALLO analogues with the carbomethoxyl in position 4′ of ring C′ replaced by a carbomethyl (KAC) and methoxy (MAC) groups were synthesized. The binding properties and consequences of binding (microtubule inhibition, abnormal polymerization, and induction of GTPase activity) were compared within the series of three ring and two ring compounds, as well as between pairs consisting of a two ring and a three ring compound with identical groups in position 4′. Binding measurements showed that the binding of KAC to the COL binding site proceeded with similar chemical characteristics as that of its two ring analogue (TKB), but with the kinetic characteristics of ALLO. The binding constant of KAC was found to be 1.9×10^6 M^{-1} and that of MAC was 4.6×10^5 M^{-1}. The binding strength of the three ring analogues in descending order was KAC > ALLO > MAC, with increments similar to the biphenyl compounds, TKB > TCB > TMB. The difference in binding affinities between the pairs of three ring and two ring molecules was invariant ($\delta\Delta G^\circ = -1.3 \pm 0.2$ kcal/mol^{-1}), showing that in all cases ring B makes only an entropic contribution by suppressing free rotation about the biaryl bond. In the case of microtubule inhibition, all three ring compounds inhibited strongly with similar potencies, even though the spread in inhibition strength between the corresponding two ring molecules was >3.3 kcal mol^{-1} of free energy. This difference was interpreted in terms of the ability of the various molecules to maintain tubulin in the proper conformation for binding in abnormal geometry to the growth end of a microtubule. This ability attains a maximal plateau value for three ring compounds, independently of the oxygen-containing group in ring C′ (or C) and is maintained for the methyl ketone whether in a two or three ring compound. The induction of the GTPase activity was found to follow in general the binding affinity, with the exception that molecules that contained a methyl ketone were stronger GTPase inducers than expected from their alignment according to binding affinity. The finding that the binding of tropolone methyl ether (ring C of COL) induced a GTPase activity shows that ring C contains the ability to induce both substoichiometric microtubule inhibition and GTPase activity. Rings A and B act only as anchors in the binding, with ring A making an energetic contribution, while the effect of ring B is only entropic. It was concluded that both microtubule assembly inhibition and induction of GTPase activity were modulated by the same postbinding conformational change in tubulin. The difference between the strengths of these activities induced by ligands reflects the difference between a narrow allosteric effect between two well-defined sites in the case of GTPase activity and a broad effect aimed at the multiple sites involved in the incorporation of a tubulin protomer into the microtubule structure. Thus, there seems to be a loose thermodynamic linkage between binding and GTPase activity, while there is none between binding and microtubule inhibition, the two phenomena being linked only kinetically.

Microtubules are ubiquitous, cytoskeletal components of eukaryotic cells. They serve as the primary structural substrates for such processes as mitosis, flagellar and ciliary-based motility, cytoplasmic transport (*2*), and cardiac cell contraction (*3*). The binding of the antimitotic drug colchicine at a specific site on the β-subunit of tubulin has several consequences: (1) microtubule assembly is inhibited; (2) tubulin acquires a low level GTPase activity directed to the GTP bound at the exchangeable site; (3) the polymerization of tubulin is reoriented to the formation of aggregates with a geometry different from that of microtubules; and (4) the circular dichroism spectrum of tubulin is slightly perturbed at 220 nm. Having a rather simple structure (see Chart 1), colchicine lends itself to a detailed submolecular examination of the role of particular groups in the induction and control of the binding to the protein and of the various observed consequences. In this way, colchicine can serve as a model for the dissection of a ligand into specific structural features that impart or control a particular biological activity. Such knowledge permits to devise modifications in the chemical

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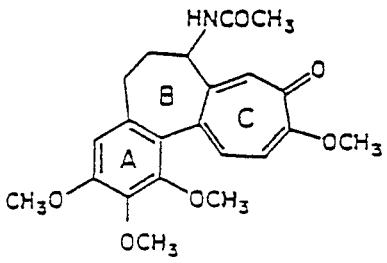
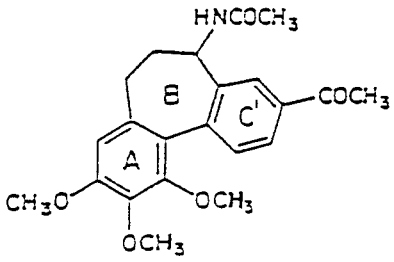
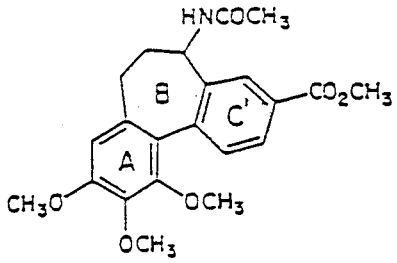
Chart 1



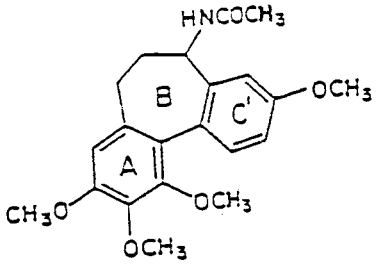
COL



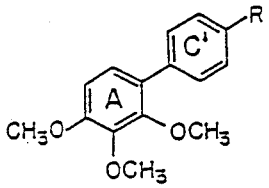
KAC



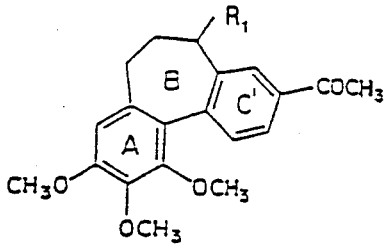
ALLO



MAC



COMPOUND	R
TCB	$COOCH_3$
TKB	$COCH_3$
TMB	OCH_3



COMPOUND	R1
NAC	NH_2
BAC	$NHCOCH_2CH_2CH_3$

features of a ligand to enhance or suppress in rational manner various aspects of its interactions with the host macromolecule.

Colchicine (COL)[1] is a three ring compound (see Chart 1). Ring A is trimethoxyphenyl. Ring C is tropolone methyl ether. The two are held in rigid spacial configuration by the seven-membered ring B. Rearrangement of ring C into carbobenzoxy phenyl ring C′ results is allocolchicine (ALLO), which has the features of a biphenyl sterically stabilized by the seven-membered ring B. Thermodynamic and kinetics analysis of the binding of COL, ALLO, and some two ring analogues of these molecules has permitted to elucidate the contribution of some structural moieties of the drug to the process of binding to tubulin (*4*−*10*). Specifically, it has been shown that the binding takes place by a bidentate mechanism. Rings A and C each binds to a specific subsite in an independent and thermodynamically noncooperative manner, as the standard free energy (ΔG_b°) of binding of COL is equal to the sum of the ΔG_b° values of the single ring (ring A and ring C) molecules, if the cratic free energy contribution is properly taken into account. Ring B appears to make no productive contribution to the binding. The noncooperativity between the bindings of rings A and C has opened the way to a systematic study of the contribution of features on ring C (or C′) to the strength of the binding, while ring A is kept intact. In these studies, it has been found that ALLO and its analogues are more amenable than COL to structural dissection because the biphenyl structure permits to isolate groups that in COL are parts of the seven-membered ring. Early results of these studies, using biphenyl (two ring) analogues of ALLO, have shown that whether position 4′ of ring C′ is occupied by a methyl ester (TCB), the group found in ALLO, or of its individual oxygen

[1] Abbreviations: AU, arbitrary units; CD, circular dichroism; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; PG buffer, 10 mM sodium phosphate, pH 7.0, 0.1 mM GTP.; COL, colchicine; ALLO, allocolchicine; KAC, 7-acetamidoallocolchinone; BAC, 7-butyramidoallocolchinone; NAC, 7-aminoallocolchinone; MAC, *N*-acetyl colchinol methyl ether; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1, 1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1, 1′-biphenyl; TMB, 2, 3, 4, 4′-tetramethoxy-1, 1′-biphenyl; TME, tropolone methyl ether; THF, tetrahydrofurane; PEG-6000, poly(ethylene)glycol 6000.

containing moieties, namely the carbonyl (methyl ketone, TKB) or the ether (methoxyl, TMB) has only a weak influence on the strength of binding (see Chart 1 for structures) (*8*, *9*).

The recent development of an equilibrium thermodynamic scheme of the in vitro inhibition of tubulin self-assembly into microtubules (*11*), based on the model of Skoufias and Wilson (*12*), has opened the way to a rigorous classification of these molecules according to their inhibitory capacity and to establish a basis for assessing linkages between the binding and inhibition phenomena (*11*). This analysis led to the unexpected result that, although the three ALLO biphenyl analogues, TCB, TKB, and TMB, bind with similar affinities to tubulin, their microtubule inhibitory capacities are vastly different: TCB is a weak stoichiometric inhibitor, TMB is a weakly substoichiometric one, and TKB is a strong substoichiometric inhibitor, with an intrinsic inhibitory capacity similar to those of COL and ALLO. Therefore, the presence of a carbonyl group on ring C′ generates a strong inhibitor if present in the form of a methyl ketone, but its inhibitory activity is suppressed by transformation into a methyl ester.[2] On the other hand, introduction of ring B into the methyl ester (TCB → ALLO) leads to a very strong inhibitory capacity. Since, by necessity, inhibition is a postbinding phenomenon, substoichiometric inhibition is the consequence of an allosteric induction of a conformational change in tubulin that prevents it from adding to the growth end of a microtubule in the geometry necessary for the closing of the microtubular cylinder, although its binding affinity to the end of a microtubule can be high (*13*).

These observations raise the following questions: if ALLO is a strong substoichiometric inhibitor, while TCB is very weak, does ring B have a specific role in the inhibition process? If so, is the effect of the drug structural moieties additive, i.e., will introduction of ring B into the methyl ketone (TKB) and methyl ether (TMB) containing biphenyls lead to the generation of much more powerful inhibitors, and if so, will such enhancement be similar in magnitude to that found for the TCB → ALLO transformation? Will the effect on the formation of the abnormal polymers be the same as that on microtubule inhibition? What will be the effects on the binding affinities to tubulin and on the induced GTPase activity? What are the linkages between these various processes? Finally, since ring C alone is sufficient to induce substoichiometric inhibition, what is its role in the induction of GTPase activity?

To answer these questions the three ring analogues of TKB and TMB were synthesized (see Chart 1). A detailed examination of the binding of these analogues (KAC and MAC) to tubulin and its consequences was undertaken. The possible role of ring B was further probed by the synthesis of two analogues of KAC that contained a polar and a nonpolar side chain on ring B, respectively. The results of these studies and the definition of the linkages between the different induced functions are the subject of this paper.

EXPERIMENTAL PROCEDURES

Chemicals. COL and podophyllotoxin were from Aldrich Chemical Co. ALLO was prepared by the base-catalyzed rearrangement of COL (*14*) as described previously (*8*). GTP (sodium salt type III) was from Sigma. [γ-^{32}P]-Labeled GTP (25 Ci/mmol) was obtained from ICN Radiochemicals (Costa Mesa, CA). [^{3}H]-Labeled GDP (12.8 Ci/mmol) was purchased from New England Nuclear. MTC was kindly provided by Dr. T. J. Fitzgerald (Florida State University, Tallahassee, FL). Ultrapure grade ammonium sulfate and sucrose were obtained from Schwarz/Mann. Ultrapure guanidine hydrochloride was purchased from United States Biochemicals, and its solution was filtered through a sintered glass funnel to remove insoluble debris before use. Bio-Gel P-100 was from Bio-Rad laboratories and Blue Dextran 2000 was from Pharmacia Fine Chemicals. Dimethyl sulfoxide was from Sigma. All other chemicals used were reagent grade.

Synthesis of Ligands. 7-Acetamidoallocolchinone (KAC) was synthesized by the method of the modified synthesis of ketones by a Grignard reaction with esters (*15*). In a reaction flask kept under a dry nitrogen atmosphere were mixed in a THF medium, 0.03 M methylmagnesium bromide (Aldrich) and 0.11 M triethylamine, freshly distilled from P_20_5. The reaction flask was kept in an ice−water bath with stirring. ALLO (0.005 M) dissolved in 150 mL of C_6H_6 plus 5 mL of THF was added during the course of 3 h. After work up of the reaction mixture, the material obtained was chromatographed twice on silica plates and recrystallized from ethyl acetate. MP: 219−220 °C. Elemental analysis, found: C, 68.93; H, 6.69; N, 3.69. Theory: C, 68.89; H, 6.57; N, 3.65. Mass spectrum: 383 (79%); 384 (22%); 324 (77%) (loss of CH_3CONH). UV maximal absorption occurred at 301 nm and minimal absorption at 265 nm in CH_3OH. The molar extinction coefficient in 0.01 M sodium phosphate buffer, pH 7.0, 1% DMSO at 300 nm is 15 540 M^{-1} cm^{-1}. IR (CCl_4): 1675 cm^{-1} (C═O ketone); 1645 cm^{-1} (C═O amide); 1595 cm^{-1} (aromatic); 1484 cm^{-1} (aromatic); 1463 cm^{-1} (aromatic); 1292 cm^{-1} [C−(C═O)−C]; 1232 cm^{-1} (C−O ether); 1106 cm^{-1} (C−O) cm^{-1}. ^{1}NMR ($CDCl_3$): δ 7.98 [dd, J = 1.7 Hz, 3 Hz, H−C (3′)]; 7.84 [dd, J = 8.0 Hz, 1.7 Hz, H−C (5′)]; 7.60 [dd, J = 80 Hz, 3.0 Hz, H−C (6′)]; 6.60 [s, H−C (5)]; 6.44 (d, J = 80 Hz, H−N); 4.91 [m, H−C (9)]; 3.94, 3.91 (s, ring A *m*-, *p*-OCH_3); 3.59 (s, ring A *o*−OCH_3); 2.64 (s, ring C ketone CH_3); 2.48 [m, H−C (7)]; 2.05 (s, amide CH_3); 1.85 [m, H−C (8)]. ^{13}C NMR ($CDCl_3$): δ 198.2 (C═O, ketone); 169.5 (C═O, amide); 153.3 (C-2); 151.2 (C-4); 140.1 (C-2′); 141.3 (C-3); 135.7 (C-4′); 134.8 (C-1′); 130.3 (C-6′); 126.9 (C-5′); 123.9 (C-1, C-6); 121.9 (C-3′); 107.8 (C-5); 61.2, 60.7, 56.1 (ring A OCH_3); 52.5 (C-9); 39.2 (C-7); 30.4 (C-8); 26.6 (ketone CH_3); 23.2 (amide CH_3).

7-Aminoallocolchinone (NAC) was prepared by keeping 7-acetamidoallocolchinone for 2 weeks at 37 °C in 4% H_2-$S0_4$ in methanol. After work up, the material was chromatographed on silica plates to give the pure amine in 85% yield. Mass spectrum: 341 (100%); 324 (84%) (loss of NH_2). UV maximal absorption occurred at 297 nm in CH_3-OH, with a molar extinction coefficient of 14 000 M^{-1} cm^{-1} at 300 nm in 0.01 M sodium phosphate buffer, pH 7.0, and 1% DMSO. IR (CCl_4): 1674 cm^{-1} (C═O ketone); 1598 cm^{-1} (aromatic); 1484 cm^{-1} (aromatic); 1463 cm^{-1} (aromatic); 1291 cm^{-1} [C−C═O)−C]; 1231 cm^{-1} (C−O ether); 1101 cm^{-1} (C−O) cm^{-1}. ^{1}NMR ($CDCl_3$): δ 8.27 [d, J = 1.5 Hz, H−C (3′)]; 8.3 (ring B H_2N); 7.91 [dd, J = 1.7 Hz,

[2] A screening examination has shown that the amide ($CONH_2$) is a very weak inhibitor.

8.0 Hz, H−C (5′)]; 7.55 [d, J = 8.0 Hz; H−C (6′)]; 6.52 [s, H−C (5)]; 4.60 [m, H−C (9)]; 3.92, 3.91 (s, ring A *m*-, *p*-OCH_3); 3.64 (s, ring A *o*−OCH_3); 2.66 (s, ring C ester CH_3); 2.40 [m, H−C (7)]; 1.8 [m, H−C (8)]. ^{13}C NMR ($CDCl_3$): δ 198.2 (C═O); 153.2 (C-2, C-4); 150.8 (C-1′); 142.6 (C-2′); 140.9 (C-3); 135.7 (C-4′); 130.2 (C-6′); 126.2 (C-5′); 123.8 (C-1, C-6); 122.8 (C-3′); 107.5 (C-5); 61.0, 59.1, 56.0 (ring A OCH_3); 50.7 (C-9); 42.0 (C-7); 30.9 (C-8); 26.6 (ketone CH_3).

7-Butyramidoallocolchinone (BAC) was synthesized from 7-aminoallocolchinone by reaction with butyric acid in CH_2Cl_2 using 1,3-dicyclohexylcarbodiimide as catalyst. After stirring overnight, the solution was filtered and worked up. The crude product was chromatographed on silica plates and recrystallized several times from 1:1 C_6H_6/hexane. Mass spectrum: 411 (89%); 412 (28%); 324 (100%) (loss of $CH_3CH_2CH_2CONH$). UV maximal absorption occurred at 301 nm in CH_3OH. Extinction coefficient in 0.01 M sodium phosphate buffer, pH 7.0, 1% DMSO was 13 560 M^{-1} cm^{-1} at 300 nm. IR (CCl_4): 1675 cm^{-1} (C═O, ketone); 1645 cm^{-1} (C═O amide); 1599 cm^{-1} (aromatic); 1484 cm^{-1} (aromatic); 1464 cm^{-1} (aromatic); 1292 cm^{-1} [C−(C═O)−C]; 1233 cm^{-1} (C−O ether); 1101 cm^{-1} (C−O). ^{1}H NMR ($CDCl_3$): δ 7.96 [d, J = 8.2 Hz, H−C (3′)]; 7.86 [dd, J = 7.4 Hz, 1.8 Hz, H−C (5′)]; 7.60 [d, J = 7.8 Hz, H−C (6′)]; 6.59 [s, H−C (5)]; 6.22 [d, J = 7.8 Hz, H−N]; 4.85 [m, H−C (9)]; 3.93, 3.91 (s, ring A *m*-, *p*-OCH_3); 3.55 (s, ring A *o*−OCH_3); 2.63 (s, ring C ketone CH_3); 2.45 [m, H−C (7)]; 1.85 [m, H−C (11)]; 1.68 [H−C (12)]; 0.96 [t, J = 7.3 Hz, H−C (13)]. ^{13}C NMR ($CDCl_3$): δ 198.1 (C═O, ketone); 172.4 (C═O, amide); 153.3 (C-2); 151.3 (C-4); 142.0 (C-2′); 141.4 (C-3); 134.7 (C-1′); 131.8 (C-4′); 130.4 (C-6′); 126.9 (C-5′); 124.0 (C-1); 123.9 (C-6); 122.0 (C-3′); 107.8 (C-5); 61.3, 61.2, 56.0 (ring A OCH_3); 52.4 (C-9); 42.0 (C-7); 39.3 (C-8); 30.4 (C-11); 26.6 (ketone CH_3); 19.1 (C-12); 13.8 (C-13).

N-Acetyl colchinol methyl ether (MAC) was prepared according to the procedure of Fernholz (*14*) from allocolchicine. The reaction residue after chromatography twice on silica plates and recrystallization from benzene gave a crystalline material. UV maximal absorption occurred at 261 nm in CH_3OH. Mass spectrum: 371 (100%), the fragmentation pattern being identical with that reported previously (16). The molar extinction coefficient was 16 800 M^{-1} cm^{-1} at 259 nm in 0.01 M sodium phosphate buffer, pH 7.0, and 1% DMSO. IR (CCl_4): 1664 cm^{-1} (C═O amide); 1611 cm^{-1} (aromatic); 1484 cm^{-1} (aromatic); 1463 cm^{-1} (aromatic); 1293 cm^{-1} (C−O); 1238 cm^{-1} (C−O); 1104 cm^{-1} (C−O); 1005 cm^{-1} (C−O ether). ^{1}H NMR ($CDCl_3$): δ 7.41 [dd, J = 9.2 Hz, 5.1 Hz, H−C (6′)]; 6.90 [dd, J = 4.9 Hz, 2.0 Hz, H−C (5′)]; 6.81 [dd, J = 4.6 Hz, 1.9 Hz, H−C (3′)]; 6.53 [s, H−C (5)]; 6.30 [d, 6.0 Hz, H−N]; 4.79 [m, H−C (9)]; 3.92, 3.88 (s, ring A *m*-, *p*-OCH_3); 3.82 (s, ring C O−CH_3); 3.51 (s, ring A *o*−OCH_3); 2.40 [m, H−C (7)]; 2.00 (s, amide CH_3); 1.65 [m, H−C (8)]. ^{13}C NMR ($CDCl_3$): 169.2 (C═O amide); 158.8 (C-4′); 152.3 (C-2); 151.1 (C-4); 141.2 (C-2′); 140.8 (C-3); 134.7 (C-1′); 131.1 (C-6′); 126.6 (C-1); 124.8 (C-6); 114.2 (C-5′); 112.8 (C-3′); 107.6 (C-5); 61.2, 60.9, 56.0 (ring A OCH_3); 55.1 (ring C OCH_3); 49.2 (C-9); 39.3 (C-7); 30.5 (C-8); 23.1 (amide CH_3).

Calf Brain Tubulin. Tubulin was prepared from fresh calf brains (1 h maximum after slaughter) by a modified Weisenberg procedure (*17*, *18*), as described previously (*19*). The protein was equilibrated in the experimental buffer by a two column procedure (*19*) and its concentration was determined spectrophotometrically at 275 nm after dilution in 6 M guanidine hydrochloride with the use of an extinction coefficient of 1.03 mg^{-1} cm^{-1} (*20*).

Binding Measurements. The binding of KAC to tubulin was measured by batch gel partition (*21*−*23*), ligand fluorescence titration, and protein tryptophan fluorescence quenching. Batch gel partition is similar to dialysis equilibrium (technically excluded due to tubulin instability during the time span required to reach equilibrium) and permits the direct determination of equilibrium binding constants under conditions of constant chemical potential. The procedure was essentially that used previously for the binding of vinblastine to tubulin (*24*, *25*). Ligand fluorescence titrations were performed on a Hitachi Perkin-Elmer 650-40 spectrofluorometer, operating in the ratio mode. The fluorescence intensities of ligand solutions in the absence of protein, practically negligible, were subtracted from those measured in the presence of protein. To obtain a value of maximal fluorescence, the titration results were plotted using the procedure of Shanley et al. (*26*), as applied previously to tubulin (*27*). The experimental data were computer fitted to a single binding site equation with a commercial graphics/curve fitting program (Sigmaplot 5.0, Jandel Scientific, Corte Madera, CA). When quenching of the protein tryptophan fluorescence was employed to measure the bindings of KAC or MAC, the fraction of sites occupied, α, was taken as equal to the fraction of the determined maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was calculated assuming a 1:1 stoichiometry from $\alpha/(1 - \alpha) = K_b[A]$ (*28*), where [A] is the free-ligand concentration. The binding of MAC to tubulin was also measured indirectly by competition with MTC, as described previously for the binding of TKB to tubulin (*9*). The fractional saturation of the binding sites by MTC was measured as a function of the total concentration of competitor, MAC, at several total concentrations of MTC. The experimental data were analyzed by using the custom-made personal computer program employed previously by Medrano et al. (*9*). The equilibrium binding constant for MTC was set equal to $K_2 = 4.6 \times 10^5$ M^{-1} (*6*).

Other Procedures. The ligand-induced GTPase activity of tubulin was assayed by measuring the hydrolysis of [γ-^{32}P]-GTP in 10 mM sodium phosphate buffer, pH 7.0, 4 mM $MgCl_2$, and 0.1 mM GTP (*19*). For k_{cat} and K_m measurements, the concentration of GTP was varied from $0.2K_m$ to $2.0K_m$. Tubulin self-assembly into microtubules was monitored turbidimetrically (*11*, *29*, *30*) at 350 nm on a Cary 118 recording spectrophotometer. Circular dichroism spectra were recorded on a computer-operated Jobin Yvon Autodichrograph Mark V instrument. The spectra were obtained and analyzed by the same procedure as described previously (*8*).

RESULTS

Binding Characteristics of KAC and MAC. The binding of the drugs to the COL binding site of tubulin was demonstrated by the almost complete inhibition of the binding of COL by either KAC or MAC, as shown in Figure

Table 1: Binding of KAC and MAC to Tubulin at 25 °C

	KAC				MAC			
method	protein (M)	$K_b \times 10^{-6}$ (M^{-1})	ΔG_b° (kcal mol^{-1})	mol of ligand/ mol of tubulin	protein (M)	$K_b \times 10^{-5}$ (M^{-1})	ΔG_b° (kcal mol^{-1})	mol of ligand/ mol of tubulin
batch gel partition	3.8×10^{-6}	(1.5 ± 0.45)	−8.42	0.86				
ligand fluorescence	7.6×10^{-6}	(2.1 ± 0.25)	−8.62	0.96				
protein fluorescence quenching	1.4×10^{-6} 3.6×10^{-6}	(2.2 ± 0.45)	−8.65	*a*	1.4×10^{-6} 3.6×10^{-6}	(5.8 ± 0.7)	−7.86	*a*
competition by MTC					4.5×10^{-6}	(3.4 ± 1.2)	−7.54	*a*

[a] Information not afforded by this procedure; a 1:1 stoichiometry assumed.

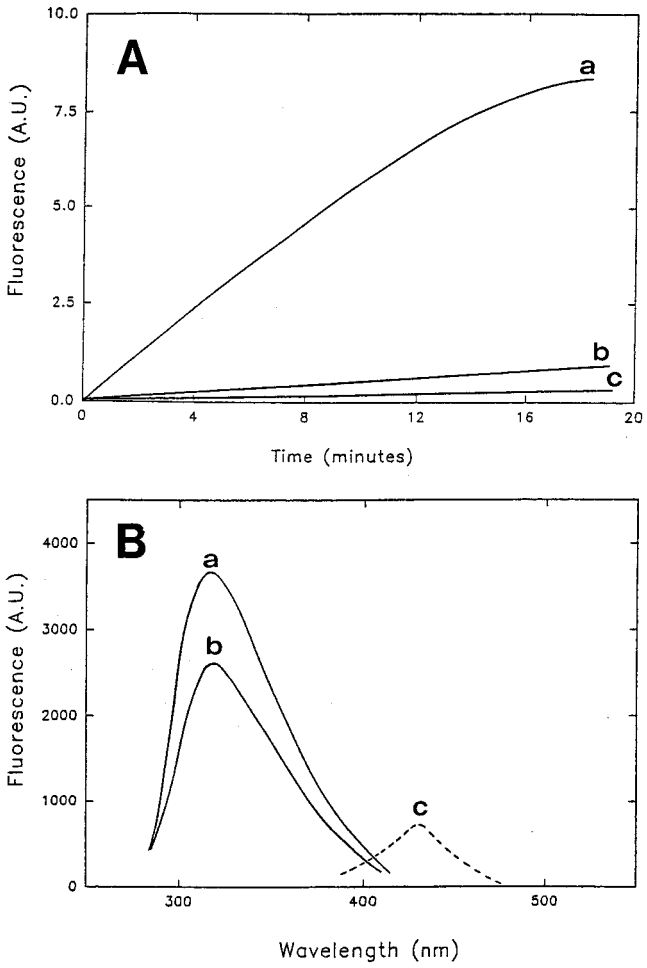


FIGURE 1: (A) Inhibition of COL binding to tubulin by KAC and MAC at 25 °C in 10 mM sodium phosphate buffer, pH 7.0, and 0.1 mM GTP. Fluorescence time courses are shown of the binding of 1×10^{-5} M COL to 7.3×10^{-6} M tubulin in the absence of analogues (a), or in the presence of 1×10^{-4} M MAC (b) and KAC (c), respectively. COL then MAC or KAC were added consecutively to the protein solution in the fluorometer cuvette at 25 °C as small volumes of concentrated solution. They were mixed simultaneously to start the reaction. The excitation and emission band widths were 10 nm using the photomultiplier gain in low. The excitation was at 357 nm and emission was monitored at 435 nm (*4*). (B) Fluorescence changes produced by the interaction of KAC with tubulin. Fluorescence emission spectra: (a) 7.3×10^{-6} M tubulin (excitation at 280 nm); (b) 7.3×10^{-6} M tubulin with 1×10^{-5} M KAC (excitation at 280 nm); (c) 1.0×10^{-5} M KAC with 7.3×10^{-6} M tubulin (excitation at 315, nm spectrum amplified 55 times). The fluorescence intensity is presented in arbitrary units (AU).

1A. The interaction of KAC with tubulin quenched the intrinsic fluorescence of a tryptophanyl group in the protein, as shown in Figure 1B by comparison of the unliganded and liganded protein emission spectra. This quenching effect is similar to that observed in the tubulin−COL (*5*) and tubulin−TKB (*9*) complexes, but different from that of the tubulin−ALLO complex (*8*). MAC quenched weakly the intrinsic fluorescence of the protein (ca. 5−8% of the protein fluorescence, not shown).

When liganded to tubulin, KAC developed fluorescence, with excitation and emission maxima at 315 and 430 nm (Figure 1B). The fluorescence is ca. 20% that of ALLO (Figure 2) and similar to that of TKB. The binding of KAC was slow, as that of ALLO, requiring 5 min to reach 95% of its maximal value (Figure 2, tracing b), whereas that of TKB reached its maximum value in less than 30 s, in agreement with Medrano et al. (*9*) (not shown). The MAC analogue did not fluoresce upon binding to the protein, just as TMB. The binding of KAC was inhibited by podophyllotoxin (PODO), the ring A subsite binder, as shown by tracings c and d of Figure 2. The inset of Figure 2 shows that KAC is displaced by 1×10^{-4} M podophyllotoxin with a half-life of the order of 98 min, i.e., more slowly than ALLO (half-life of 33 min). That for TKB is less than 30 s (*9*).

Binding Equilibrium Parameters. The binding equilibrium constants of KAC and MAC to tubulin were measured by several techniques based on different basic principles, to keep the results independent of the procedure employed. The binding isotherm of KAC to tubulin at pH 7.0, 25 °C, determined by batch gel partition, is shown in Figure 3. Analysis of the data by a Scatchard plot led to a binding equilibrium constant, $K_b = (1.5 \pm 0.45) \times 10^6$ M^{-1} and 0.86 site per tubulin dimer, which was independent of the GTP concentration between 0.1 and 1 mM. As a control, the binding of ALLO was measured under the same conditions, giving values of $K_b = (7.5 \pm 2.2) \times 10^5$ M^{-1} and a stoichiometry of 0.68 sites per tubulin dimer, in agreement with previous measurements (*8*, *31*). The results of ligand fluorescence titration for KAC and ALLO under identical conditions are shown in the form of Scatchard plots in Figure 4A (solid and open circles, respectively). The K_b values obtained were $(2.1 \pm 0.25) \times 10^6$ M^{-1} for KAC and $(8.5 \pm 0.6) \times 10^5$ M^{-1} for ALLO. The results of binding measurements by the protein tryptophan fluorescence quenching approach for KAC (squares) and MAC (triangles) are shown in Figure 4B. The corresponding values of K_b were $(2.2 \pm 0.45) \times 10^6$ M^{-1} for KAC, and $(5.8 \pm 0.7) \times 10^5$ M^{-1} for MAC. The binding of MAC to the COL site of tubulin was also examined by competition with the reference fluorescent ligand MTC. These results, shown in Figure 5, gave $K_b = (3.4 \pm 1.2) \times 10^5$ M^{-1}. A previous study (*32*), using different experimental conditions and techniques, showed a

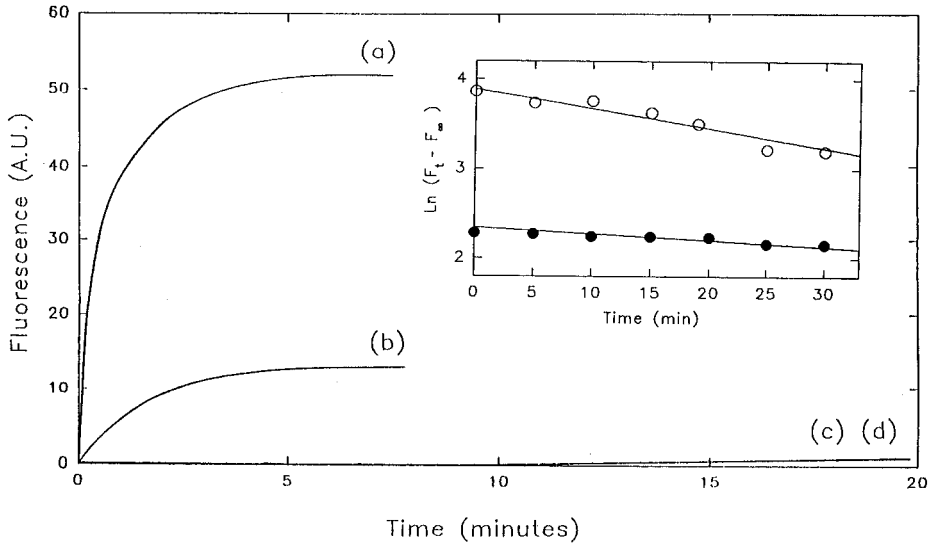


FIGURE 2: Fluorescence time course of the binding of ALLO and KAC to tubulin. The reactions were started by addition of the ligands to the protein solution: (a) 2×10^{-5} M ALLO and 5×10^{-6} M tubulin; (b) 2×10^{-5} M KAC and 5×10^{-6} M tubulin. Tracings c and d are the same as a and b, except that podophylotoxin (1×10^{-4} M) was added 4 min before ALLO or KAC, respectively. The excitation wavelength was 315 nm for ALLO and KAC, while the emission was monitored at 390 nm (ALLO) and 430 nm (KAC). (Inset) First-order plot for the displacement of ALLO (○) and KAC (●) by 1×10^{-4} M podophyllotoxin. The fluorescence intensity is given in arbitrary units.

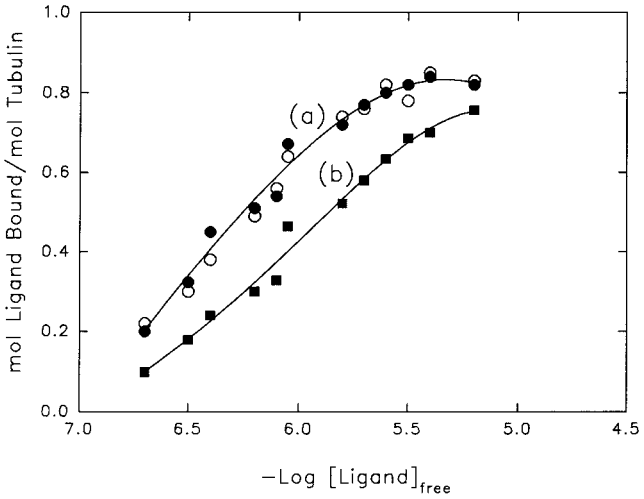


FIGURE 3: Binding isotherms of KAC (circles) and ALLO (squares) to tubulin at 25 °C obtained by the batch gel partition procedure (see Materials and Methods) in 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM GTP (●, ■) or 1 mM GTP (○). The points were obtained by UV absorbance (300 nm for KAC and 315 nm for ALLO) of the binding measurements. The protein concentration was 3.8×10^{-6} M. The solid lines are fits to the absorbance measurements [(a) $K_b = (1.5 \pm 0.45) \times 10^6$ M^{-1}, $n = 0.86$; (b) $K_b = (7.5 \pm 2.2) \times 10^5$ M^{-1}, $n = 0.68$], obtained from Scatchard plots of the data.

similar order of magnitude for the association constant of MAC to tubulin. A summary of the results obtained by the different procedures for KAC and MAC is presented in Table 1. Averaging of the apparent standard free energy changes, ΔG_b°, calculated from the binding equilibrium constants obtained by the different procedures (at 25 °C in PG buffer pH 7.0), resulted in values of −8.56 kcal mol^{-1} and −7.70 kcal mol^{-1} for KAC and MAC, respectively. This corresponds to equilibrium binding constants, $K_b = (1.9 \pm 0.3) \times 10^6$ M^{-1} for the three ring methyl ketone, KAC, and $K_b = (4.6 \pm 1.5) \times 10^5$ M^{-1} for for the three ring methoxy compound, MAC.

With the aim of probing further the effects of substituents in ring B on the binding to tubulin, two KAC derivatives were synthesized, in which the $HNCOCH_3$ group on ring B of KAC was replaced, in turn, by a $NH-CO-CH_2-CH_2-CH_3$ and a NH_2 group, namely a nonpolar and a polar group. Both of these compounds elicited fluorescence similar to KAC upon binding to tubulin (not shown). Scatchard plots of fluorescence titration results (Figure 4A) showed that the butyryl compound (BAC) (solid squares) binds somewhat more strongly to tubulin than KAC, [$K_b = (3.0 \pm 0.34) \times 10^6$ M^{-1}]. By contrast, the amino compound (NAC) (open squares) displayed a much weaker affinity than KAC [$K_b = (2.0 \pm 0.2) \times 10^5$ M^{-1}].

Inhibition of Microtubules. Substoichiometric amounts of KAC, BAC, NAC, and MAC were found to inhibit the in vitro microtubule assembly. As shown in Figure 6, a 50% reduction of the turbidity generated by the self-assembly of $(2.3-2.5) \times 10^{-5}$ M tubulin required 3.7×10^{-7} M KAC, 6.0×10^{-7} M BAC, 6.7×10^{-7} M MAC, and 3.0×10^{-6} M NAC. Plots of these results as a function of the total ligand-to-tubulin ratio, shown in Figure 7, showed that a 50% reduction of turbidity occurred at ratios of 0.015, 0.024, 0.029, and 0.12 for KAC, BAC, MAC, and NAC, respectively. Comparison of these ratios with that measured for COL (triangles of Figure 7) revealed that KAC is phenomenologically a stronger microtubule assembly inhibitor than COL. Introduction of a butyryl group on ring B of KAC to create BAC lowered somewhat its microtubule inhibitory potency. Studies carried out in cell cultures, however, indicate that BAC and KAC are equally strong arresters of mitosis and stronger than COL by 2 orders of magnitude[3] (data not shown). It has also been reported that MAC is a stronger mitotic arrester than COL (*1*).

[3] In an independent screening carried out at the National Cancer Institute, the GI_{50}s (concentration at which the cell growth is slowed 50%) of KAC and BAC were about 100 times lower than those of COL against most of the 60 cell lines tested.

Table 2: Interaction of Colchicine Analogues with Tubulin at 37 °C as Determined from the Assembly Data

analogue	K_b (M^{-1})	ΔG_b° [b] (kcal mol^{-1})	K_i (M^{-1})	ΔG_i° [c] (kcal mol^{-1})	protein[d] (M)	r[e] (%)	inhibition
COL	1.6×10^7 (37 °C)[a]	−10.2	$(2.2 \pm 0.2) \times 10^6$ [a]	−9.00[a]	$(1.7-2.2) \times 10^{-5}$ [a]	1.9 ±0.2[a]	substoichiometric
ALLO	8.0×10^5 (25 °C)[f]	−8.05	$(2.1 \pm 0.5) \times 10^6$ [a]	−8.97[a]	$(1.6-2.4) \times 10^{-5}$ [a]	1.9 ±0.5[a]	substoichiometric
TKB	1.9×10^5 (25 °C)[a]	−7.20	$(2.1 \pm 0.3) \times 10^6$ [a]	−8.97[a]	$(1.6-3.0) \times 10^{-5}$ [a]	2.4 ±0.4[a]	substoichiometric
KAC	1.9×10^6 (25 °C)	−8.56	$(2.7 \pm 0.4) \times 10^6$	−9.12	$(2.3-2.5) \times 10^{-5}$	1.4 ± 0.2	substoichiometric
TMB	8.2×10^4 (25 °C)[a]	−6.70	$(9.6 \pm 1.4) \times 10^4$ [a]	−7.07[a]	$(1.8-2.6) \times 10^{-5}$ [a]	17 ± 2[a]	weak substoichiometric
MAC	4.6×10^5 (25 °C)	−7.72	$(1.7 \pm 0.3) \times 10^6$	−8.84	$(2.2-2.4) \times 10^{-5}$	2.3 ± 0.4	substoichiometric
BAC[g]	3.0×10^6 (25 °C)	−8.83	$(1.6 \pm 0.3) \times 10^6$	−8.80	$(2.4-2.6) \times 10^{-5}$	2.3 ± 0.3	substoichiometric
NAC[g]	2.0×10^5 (25 °C)	−7.23	$(3.7 \pm 0.7) \times 10^5$	−7.90	2.5×10^{-5}	7.9	substoichiometric
TCB	1.0×10^5 (25 °C)[a]	−6.82	$<10^4$ [a]	>−5.6	$(2.0-2.4) \times 10^{-5}$ [a]	40 ± 9[a]	stoichiometric
MTC	3.6×10^5 (37 °C)[a]	−7.88	$(5.6 \pm 0.2) \times 10^5$ [a]	−8.15[a]	$(1.8-3.0) \times 10^{-5}$ [a]	5.8 ± 0.2[a]	substoichiometric
TME	3.5×10^2 (37 °C)[a]	−3.61	$(8.7 \pm 0.4) \times 10^5$ [a]	−8.42[a]	$(2.3-2.6) \times 10^{-5}$ [a]	4.2 ± 0.8[a]	substoichiometric

[a] Reference 11. [b] Standard free energy of binding of the drug to the $\alpha\beta$ tubulin dimer. [c] The standard free energy of inhibition was calculated from the K_i values. [d] Protein concentration at which the K_i values were calculated. [e] Liganded tubulin at 50% turbidity. The ± values reflect the spread between individual experiments; they do not take into account the uncertainties of the K_b values. [f] Reference 31. [g] KAC analogues where ring B $NHCOCH_3$ has been replaced by a $NHCOCH_2CH_2CH_3$ and a NH_2 group, respectively.

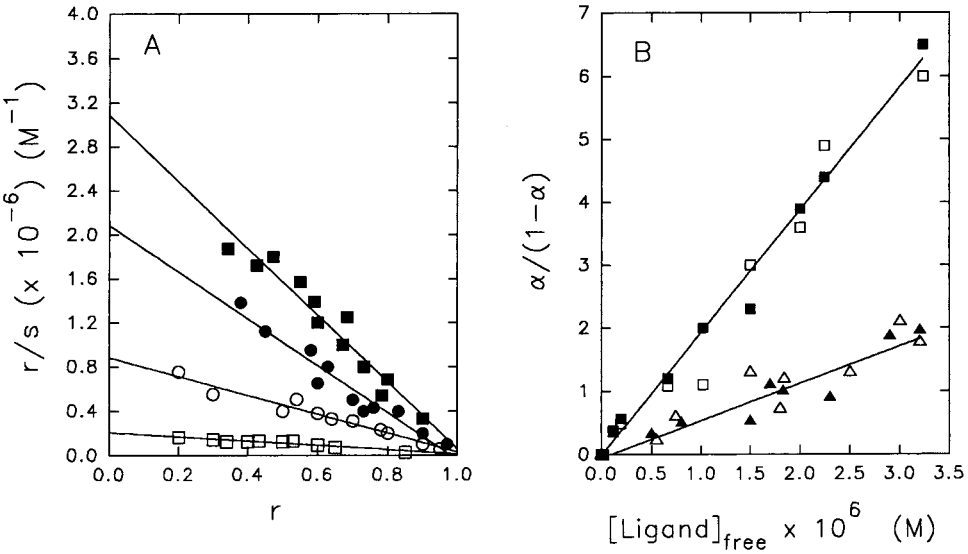


FIGURE 4: (A) Scatchard plot for the bindings of KAC (●), BAC (■), NAC (□), and ALLO (○) to tubulin as determined by ligand fluorescence titration at 25 °C in PG buffer. The concentration of protein was 7×10^{-6} M in each case. The excitation wavelength for the analogues was 315 nm, whereas the emission was monitored at 430 nm for KAC and 410 nm for BAC and NAC, respectively. (B) Protein tryptophan fluorescence quenching titration of the binding of KAC to (□) 1.4×10^{-6} M and (■) 3.6×10^{-6} M tubulin and the binding of MAC to (△) 1.4×10^{-6} M and (▲) 3.6×10^{-6} M tubulin at 25 °C in PG buffer. The excitation monochromator was set at 280 nm and the emission was recorded at 295 nm using 5 and 10 nm slits for the excitation and emission, respectively. In panel A, r and s are fraction of bound drug and free drug concentration, respectively.

Comparison of the microtubule inhibitory potency using the total drug levels needed to reduce tubulin self-assembly by 50% is a procedure subject to great uncertainty, because the level of inhibition is a function both of the strength of binding of the drug to the protein and of the ability of the resulting complex to stop microtubule growth. A quantitative comparison of the inhibitory capacities of drugs requires a deconvolution of the two processes. This can be done by application of the recently proposed mechanism that gives a quantitative description of the inhibition process (*11*), based on the finding by Skoufias and Wilson (12) that the microtubule growth blocking entity is the tubulin−COL complex. This mechanism is given by reaction Scheme 1:

Scheme 1

$$M_{n-1}TA \overset{K_i}{\rightleftharpoons} TA + M_{n-1} \overset{K_b}{\rightleftharpoons} A + T + M_{n-1} \overset{K_g}{\rightleftharpoons} M$$

where K_g is the normal microtubule growth constant, equal to Cr^{-1} (Cr is the critical concentration in the absence of drugs), K_b is the binding constant of the drug to tubulin; K_i is the microtubule inhibition constant, which is the binding constant of the tubulin−drug complex to the end of a growing microtubule and, hence, a measure of the intrinsic microtubule inhibitory capacity of the drug, T is free tubulin, A is free drug, M_{n-1} is microtubule before elongation and M is microtubule after addition of one tubulin $\alpha\beta$ protomer. In terms of this scheme, the fraction of turbidity measured in the presence of an inhibitor relative to that in its absence can be used to deduce K_i, i.e., the strength of the inhibitory capacity of the drug. Scheme 1 leads to eq 1 (*11*).

$$\text{fraction} = \frac{1}{1 + K_b K_i K_g^{-1}[A]} - \frac{K_b K_g^{-1}[A]}{(T_{total} - Cr)(1 + K_b K_i K_g^{-1}[A])} \quad (1)$$

The data of Figure 6 were analyzed in this manner, and the deduced values of K_i are listed in Table 2. These values

Table 3: Ligand Induced GTPase of Tubulin

ligand	maximal rate (min^{-1})[a]	$k_{cat}^{apparent}$ (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
COL	0.0095 ± 0.000 46	$2.0 \times 10^{-4} \pm 4.6 \times 10^{-6}$ [b]	9.0 ± 2[b]	2.22×10^{1} [b]
KAC	0.0084 ± 0.000 76	$1.6 \times 10^{-4} \pm 1.1 \times 10^{-5}$	9.1 ± 2	1.76×10^{1}
KAC	0.0285 ± 0.0034[c]	ND[d]	ND	ND
KAC	0.102 ± 0.0083[c]	ND	ND	ND
BAC	0.0084 ± 0.000 58	ND	ND	ND
NAC	0.0077 ± 0.000 64	ND	ND	ND
ALLO	0.0070 ± 0.000 63	$1.3 \times 10^{-4} \pm 4.5 \times 10^{-6}$ [b]	8 ± 0.6	1.66×10^{1} [b]
MAC	0.0064 ± 0.000 60	$1.4 \times 10^{-4} \pm 1.3 \times 10^{-5}$	9.4 ± 3	1.48×10^{1}
MAC	0.0211 ± 0.0033[c]	ND	ND	ND
MAC	0.0713 ± 0.0061[c]	ND	ND	ND
MTC	0.0060 ± 0.000 59	$1.8 \times 10^{-4} \pm 1.8 \times 10^{-5}$ [b]	12 ± 3[b]	1.50×10^{1} [b]
TCB	0.0057 ± 0.000 68	$1.3 \times 10^{-4} \pm 1.6 \times 10^{-5}$ [b]	10 ± 1.7[b]	1.30×10^{1} [b]
TMB	0.0043 ± 0.000 56	$(9.5 \pm 1.0) \times 10^{-5}$[b]	15 ± 2.4[b]	0.63×10^{1} [b]
TKB	0.0068 ± 0.000 74	$1.3 \times 10^{-4} \pm 1.3 \times 10^{-5}$ [b]	11 ± 2.1[b]	1.14×10^{1} [b]
TME	0.000 47 ± 0.000 19[e]	ND	ND	ND

[a] Apparent maximal rate of GTP hydrolysis from initial velocity experiments in 10 mM sodium phosphate buffer, pH 7.0, and 4 mM $MgCl_2$ at a fixed GTP concentration of 0.1 mM. The ligand and protein concentration were 200 and 2 μM, respectively. [b] Reference 19. [c] Buffer containing 3.4 M glycerol and 1% w/v PEG-6000, respectively. [d] Not determined. [e] Measurements carried out in 1.2 mM TME and 2 μM tubulin.

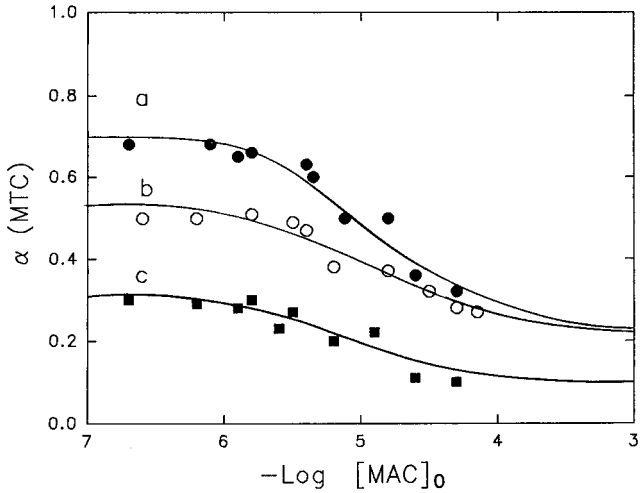


FIGURE 5: Competition of MAC with the reference ligand MTC for the binding to tubulin. The binding of MTC was measured fluorometrically as a function of total competitor concentration, and the results were analyzed as described in Experimental Procedures. The total tubulin concentration was 4.5×10^{-6} M, and the total MTC concentrations were (a) 1×10^{-5} M, (b) 0.5×10^{-5} M, and (c) 0.3×10^{-5} M. The excitation and emission wavelengths were 350 and 423 nm, respectively. The experimental data were treated as described in Experimental Procedures.

were combined with those of K_b and K_g to construct the theoretical inhibition curves presented in Figure 8 in the form of isotherms as a function of free ligand concentration. The good fit of the experimental data for all four new microtubule assembly inhibitors shows that this simple inhibition model can be used to compare their inhibitory capacity with that of COL and its analogues. The value of the inhibition constant (K_i) for KAC is $(2.7 \pm 0.4) \times 10^6$ M^{-1} (see Table 2). This value is higher than those of its biphenyl analogue TKB [$K_i = (2.1 \pm 0.3) \times 10^6$ M^{-1}], ALLO [$K_i = (2.1 \pm 0.5) \times 10^6$ M^{-1}], and COL [$K_i = (2.2 \pm 0.2) \times 10^6$ M^{-1}] (*11*). Thus, intrinsically, KAC is a stronger inhibitor than COL although the binding constant of COL to tubulin is 1 order of magnitude greater than that of KAC (Table 2). The methoxy analogue, MAC, with $K_i = (1.7 \pm 0.3) \times 10^6$ M^{-1}, is a weaker inhibitor than ALLO and KAC, but much stronger than its biphenyl analogue, TMB [$K_i = 9.6 \pm 1.3) \times 10^4$ M^{-1}]. Replacement of the $NHCOCH_3$ group in ring B of KAC, in turn, by a $NHCOCH_2CH_2CH_3$ (BAC) and a NH_2 group (NAC) led to a slight weakening of inhibition for BAC [$K_i = (1.6 \pm 0.3) \times 10^6$ M^{-1}], and drastic weakening for NAC [$K_i = (3.7 \pm 0.7) \times 10^5$ M^{-1}].

The inhibition results were analyzed further by the independent procedure of calculating the extent of tubulin liganding at 50% reduction of turbidity, *r*, as described previously (*11*). The results listed in Table 2 show that all four new analogues are substoichiometric inhibitors. In fact, KAC ($r = 1.4\%$) is the strongest inhibitor by this criterion as well, since for COL and ALLO, $r = 1.9\%$, whereas MAC and BAC are essentially equal to TKB ($r = 2.3\%$). These values mean that, in the case of KAC, at 50% inhibition, one tubulin is liganded for each 71 free; for COL and ALLO the ratio is 1:52. For MAC and BAC it is 1:42. On the other hand, NAC is a much weaker substoichiometric inhibitor, there being only 12 tubulin molecules free for each liganded.

Abnormal Polymers. When COL binds to tubulin, it reorients the self-assembly process of this protein to form polymers with the thermodynamic characteristics of microtubule assembly, but an aberrant geometry (*13*, *33*, *34*). As shown in Figure 9, panels A−C an excess of KAC, BAC, or NAC over tubulin induced an increase of turbidity in 10 mM sodium phosphate buffer, pH 7.0, 16 mM $MgCl_2$, and 1 mM GTP in the absence of glycerol, when the solution was heated to 37 °C. This was fully reversed by cooling to 10 °C. MAC did not induce the abnormal polymerization at 37 °C; the observation was that of aggregation that was not reversed by cooling at 10 °C (not shown). Abnormal polymerization reversed by cooling to 10 °C could be induced with this analogue by raising the temperature to 42 °C (Figure 9D). This behavior is similar to that of its biphenyl analogue, TMB (*7*). For all four analogues, the process was characterized by a lag time and a critical concentration, characteristic of nucleated cooperative self-assembly (*35*). The values of the critical concentration were 0.53, 0.50, 1.2, and 1.0 mg ml^{-1} tubulin in the presence of KAC, BAC, NAC (at 37 °C), and MAC (at 42 °C), respectively (see insets to Figure 9). These values correspond to growth constants $K_g = Cr^{-1}$ (*30*, *36*) of 2.1×10^5 M^{-1} for KAC, 2.2×10^5 M^{-1} for BAC and 0.9×10^5 M^{-1} for NAC at 37 °C, and 1.1×10^5 M^{-1} for MAC at 42 °C, i.e., values similar to that of COL (*34*).

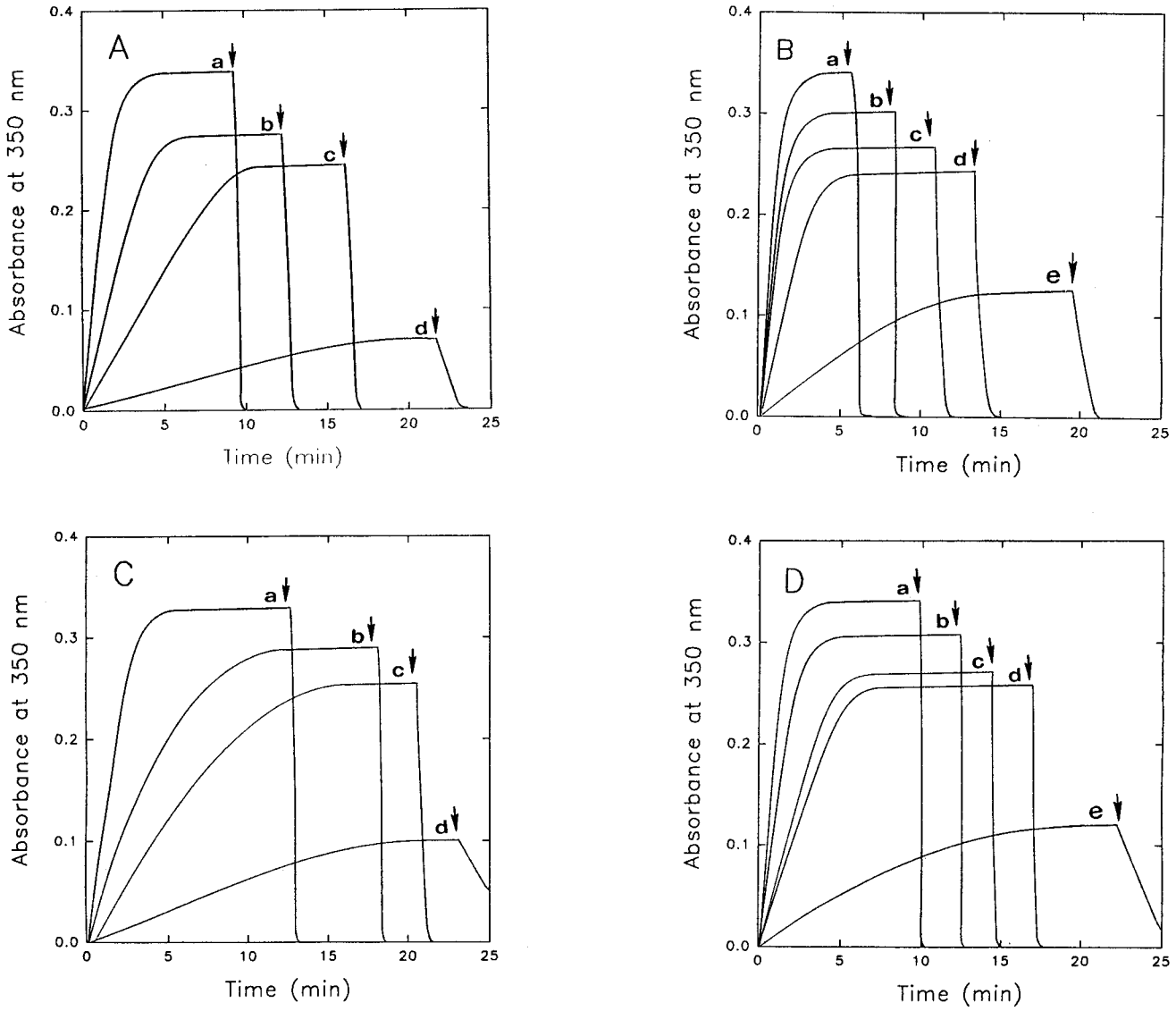


FIGURE 6: Representative traces of the effects of KAC, BAC, NAC, and MAC on the turbidity time course of the in vitro microtubule assembly. The protein was preincubated with the ligand for 30 min at 20 °C before initiation of the assembly. The reaction was started by warming the solution in the assembly buffer (10 mM sodium phosphate, pH 7.0, 16 mM $MgCl_2$, 3.4 M glycerol, and 1 mM GTP) from 10 to 37 °C; the arrows indicate cooling of the samples at 10 °C. (A) (a) 2.4×10^{-5} M tubulin; (b−d) same, with 1.0×10^{-7} M, 1.48×10^{-7} M, and 1.2×10^{-6} M KAC. (B) (a) 2.5×10^{-5} M tubulin; (b−d) same, with 1.25×10^{-7} M, 2.5×10^{-7} M, 3.75×10^{-7} M, and 1.0×10^{-6} M BAC. (C) (a) 2.3×10^{-5} M tubulin; (b−d) same, with 9.2×10^{-8} M, 2.1×10^{-7} M, and 5.3×10^{-6} M MAC. (D) (a) 2.5×10^{-5} M tubulin; (b−d) same, with 5.8×10^{-7} M, 6.5×10^{-7} M, 1.25×10^{-6} M, and 3.2×10^{-6} M NAC.

Ligand-Induced GTPase. Another consequence of COL binding is the induction of GTPase activity in tubulin (*37*, *38*). Measurements of the GTPase activity induced by the analogues are summarized in Table 3. The ligands KAC and MAC induced a GTPase activity in tubulin with an apparent k_{cat} of $(1.6 \times 10^{-4}) \pm (1.1 \times 10^{-5})$ s^{-1} for KAC and $(1.4 \times 10^{-4}) \pm (1.2 \times 10^{-5})$ s^{-1} for MAC and a K_m for GTP of 9.1 ± 2 μM for KAC and 9.4 ± 3 μM for K_m for MAC. A comparison of the apparent maximal rate of GTP hydrolysis (at a fixed GTP concentration) by tubulin liganded to several COL analogues is shown in Table 3, column 2. The parallel between binding affinity and GTPase induction seems to be, in general, maintained, although the high values of NAC and TKB suggest some specificity of the ketone. The striking result is that TME, which is ring C of COL that binds very weakly to tubulin ($K_b = 3.5 \times 10^2$ M^{-1}) (*5*), elicited a significant GTPase activity, 5% of that obtained with the tubulin−COL complex. While the large experimental error precludes an exact definition of the magnitude of the GTPase induction by TME, it is clear that the binding of ring C alone is sufficient to induce this activity.

In earlier studies (*10*, *27*), it had been shown that the difference between the apparent equilibrium binding constant, K_b, deduced from the titration of GTPase activity and that measured by physical techniques permitted to characterize a postdrug binding conformational shift between GTPase inactive and active tubulin−drug complexes, expressed by equilibrium constant K_3 in Scheme 2 (*27*):

Scheme 2

$$\text{L} + \text{T-GTP} \underset{(1)}{\overset{K_1}{\rightleftharpoons}} \text{L-T-GTP} \underset{(2)}{\overset{K_2}{\rightleftharpoons}} \text{L-T°-GTP} \underset{(3)}{\overset{K_3}{\rightleftharpoons}} \text{L-T}^{\text{active}}\text{-GTP}$$

where L is ligand, T is tubulin, and L−T° is the end product

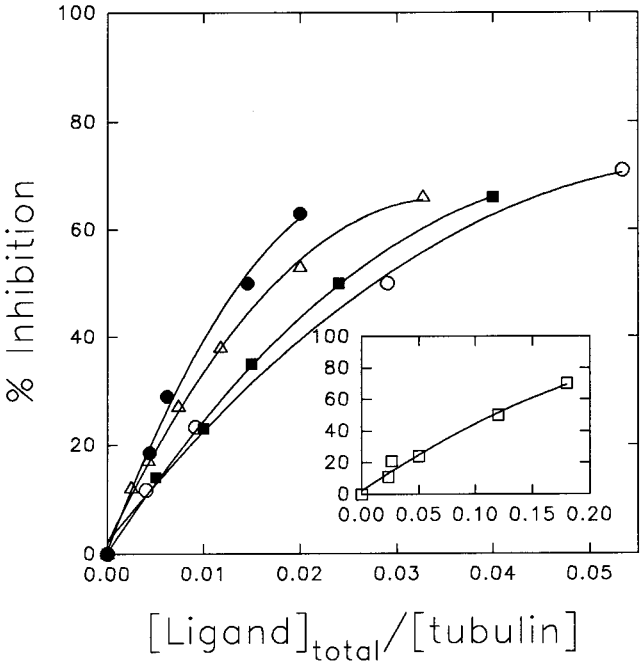


FIGURE 7: Fraction of the reduction of the plateau absorbance values, expressed as percent inhibition, as a function of the ratio of the total drug concentration to the protein concentration. KAC (●), COL (△), BAC (■) and MAC (○). (Inset) NAC.

of drug binding measured by physicochemical techniques (*10*, *27*); L−T^{active}-GTP is the GTPase-active liganded−tubulin complex detected by enzyme activity titration. The first two steps (K_1 and K_2) correspond to the binding process described by Garland (*39*). The titration of KAC binding to tubulin by GTPase activity measurements, presented in the form of a Scatchard plot in Figure 10, gave an apparent equilibrium binding constant, $K_b = (6.2 \pm 2.2) \times 10^5$ M^{-1}, which is three times smaller than that measured by fluorescence titration. Combination of this value with that obtained by fluorescence titration gave the value of K_3 and, hence, the fraction of liganded tubulin present in the GTPase active conformation (*27*), which was found to be 10.2% for KAC. The resulting calculated value of $k_{cat}^{intrinsic}$, i.e., $V_{max} \times [L-T^{active}]^{-1}$, of 6.7×10^{-4} s^{-1} was similar to that obtained with MTC (*27*). Additions of glycerol (3.4 M) and PEG-6000 (1% w/v) activated both the KAC- and MAC-induced GTPase activities 3.4- and 12-fold, respectively (Table 3). This is similar to the effects found in the presence of ALLO and MTC (*27*).

Effect of KAC Binding on the Circular Dichroism Spectrum. Similarly to COL, the binding of KAC to tubulin induced a small perturbation of the protein circular dichroism spectrum in the far-UV. Figure 11 shows the far-ultraviolet circular dichroism spectra of tubulin in the absence (tracing a) and in the presence (tracing b) of KAC, as well as the calculated difference spectrum (inset). The increment in ellipticity in the presence of KAC had a magnitude of −653 ± 294 deg cm^2 dmol^{-1} near 220 nm. The ratio $[\theta](220)/[\theta](210)$ changed from 1.017 for unliganded tubulin to 1.083 in the presence of KAC. This small change induced by KAC is similar to values previously reported for tubulin liganded to COL (34), MTC (*6*), ALLO (*8*), TKB (*9*), and TME (*34*) and much greater than that for TCB (*8*). This is consistent with the perturbation of a tryptophan residue in tubulin that could also be involved in the protein fluorescence quenching by the analogues.

DISCUSSION

Following the observation that introduction of ring B into the carbomethoxy biphenyl analogue of colchicine (TCB) to form ALLO is accompanied by a dramatic change of the microtubule inhibitory capacity of the drug, while the effect on drug binding is simply entropic, a systematic study has been carried out of the effects of the introduction of ring B into biphenyls on the interactions of these molecules with tubulin. The questions asked were essentially (1) is there additivity of the effects of the structural moeities of these ligands on the various phenomena engendered by their interactions with tubulin? (2) Is there specificity of any structural moieties in the induction and control of particular properties of the protein−drug complex? (3) What are the linkages, if any, between the various manifestations of the interaction between tubulin and the colchicine family of molecules? Answers to these questions in a systematic and rational manner require a comparison of the full sets of three ring and two ring compounds. To this end, the three ring compounds with a methyl ketone (KAC) and a methyl ether (MAC) in position 4′ of ring C′ were synthesized. This has led to the extension of the noncooperative nature of the binding process to detailed features of drug structure. For microtubule inhibition and induction of GTPase activity, the consequences are more complex. It must be noted that, ring A being common to all the molecules, all the observed effects must be caused by differences within ring C.

Binding Characteristics and Affinities. A detailed examination of the binding process to tubulin of the ALLO analogue that carries a methyl ketone in position 4′ (KAC) has shown that this process has characteristics of the bindings both of ALLO and of the two ring methyl ketone (TKB). Chemically, the nature of the interactions seems identical whether the ketone is a two ring or a three ring compound: the two quench the fluorescence of a protein tryptophan with very similar spectral characteristics, which differ, however, from those of the ALLO−tubulin complex. That these various ligands interact with the same Trp residue is confirmed by the essential identity of the perturbation of the tubulin CD spectrum at 220 nm. Kinetically, however, the three ring ketone behaved like ALLO, both its binding and displacment by PODO being slow. For the two ring analogue, TKB, both processes are rapid. This indicates that in the KAC-TKB pair, the influence of ring B on the binding kinetics is the same as in the ALLO-TCB pair. This differs, however, from the COL-MTC pair, for which removal of ring B renders the binding very rapid, while the displacement by PODO remains slow.

Comparison of the affinities of the several analogues for the binding site on tubulin shows that the relative affinities are determined strictly by the chemical nature of the groups on ring C′ (or C). Thus, the order of decreasing affinities of the two ring compounds is MTC > TKB > TCB > TMB; for the three ring compounds, it is COL > KAC > ALLO > MAC. The individual increments in ΔG_b° along the two series are identical within experimental error. The difference in affinity between the pairs of three and two ring molecules is essentially invariant (−1.3 ± 0.2 kcal mol^{-1} of standard

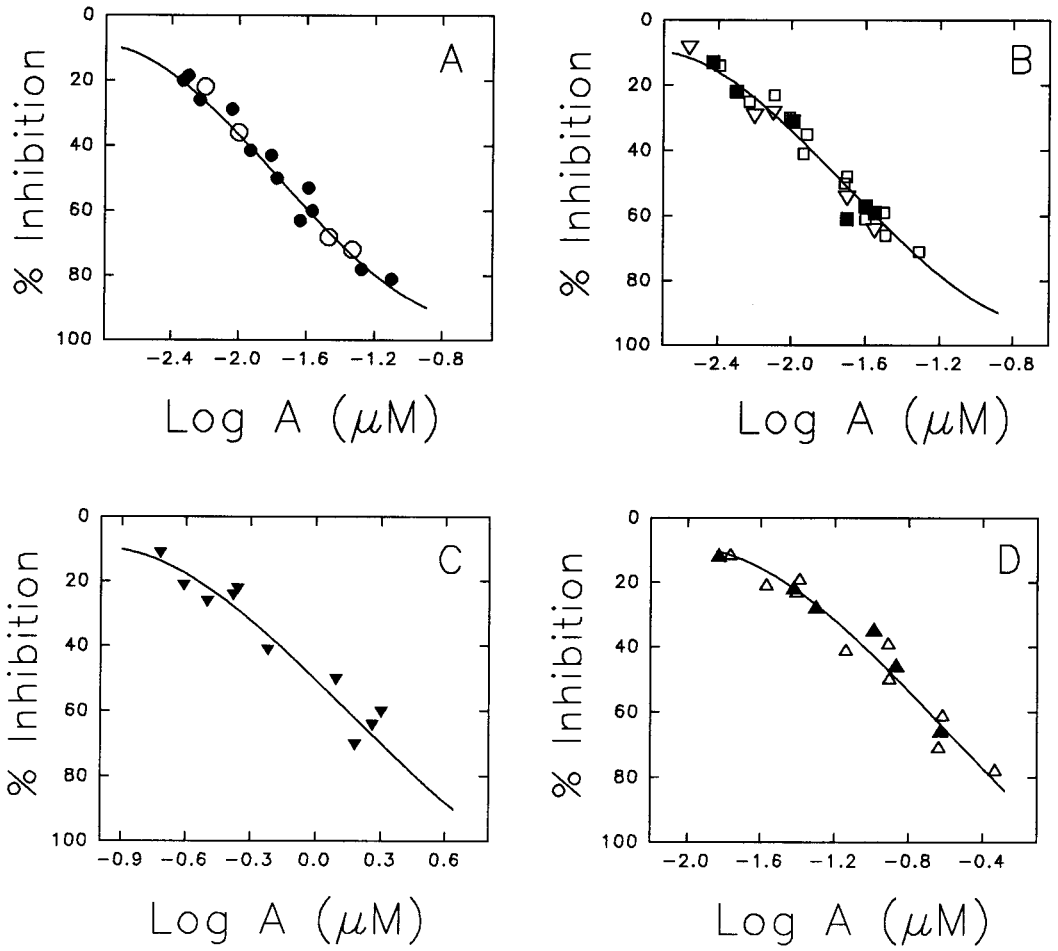


FIGURE 8: Inhibition of tubulin self-assembly as a function of free drug concentration ([A]), plotted as isotherms. (A) Effect of KAC; (● and ○) 2.3×10^{-5} and 2.5×10^{-5} M tubulin, respectively. (B) Effect of BAC; (■, □, ▽) 2.4×10^{-5}, 2.5×10^{-5}, and 2.6×10^{-5} M tubulin, respectively. (C) Effect of NAC; (▼) 2.5×10^{-5} M tubulin. (D) Effect of MAC; (▲ and △) 2.2×10^{-5} and 2.4×10^{-5} M tubulin, respectively. Free drug concentrations were calculated from the total amount of drug added, using the known equilibrium binding constants of the drugs to unassembled tubulin and setting the concentration of unliganded unassembled tubulin equal to Cr (*11*). The solid lines are the theoretical curves obtained by simultaneous fitting of the experimental values of K_b, K_i, and protein concentrations, T, at different inhibition fractions, to eq 1. The averaged values of K_i and protein concentrations were employed in the fitting using a constant K_g^{-1} value (1.0×10^{-5} M). The parameters for each drug are as follows: KAC, $K_b = 1.9 \times 10^6$ M^{-1}, $K_i = 2.7 \times 10^6$ M^{-1}, $T = 2.4 \times 10^{-5}$ M; BAC, $K_b = 3.0 \times 10^6$ M^{-1}, $K_i = 1.6 \times 10^6$ M^{-1}, $T = 2.5 \times 10^{-5}$ M; NAC, $K_b = 2.0 \times 10^5$ M^{-1}, $K_i = 3.7 \times 10^5$ M^{-1}, $T = 2.5 \times 10^{-5}$ M; MAC, $K_b = 4.6 \times 10^5$ M^{-1}, $K_i = 1.7 \times 10^6$ M^{-1}, $T = 2.3 \times 10^{-5}$ M.

free energy). This establishes firmly that the binding affinity of the three ring compounds is a simple sum of the intrinsic affinity of each A−C (or C′) compound with an invariant nonspecific contribution of ring B, which is the entropy of the elimination of free rotation about the biaryl bond (*6*, *40*). Ring B can be used, however, to introduce specificity by modifying its side chain. In the current study, that chain has been modified in two ways: (i) by replacement of the acetyl residue by the nonpolar butyryl residue (BAC); (ii) by elimination of the acetyl, which leaves a positively charged amine (NAC). The two have opposite effects. BAC binds to tubulin more strongly than KAC by −0.27 kcal mol^{-1} of standard free energy. The amine, NAC, suffers a large decrease in binding affinity, as ΔG_b°(NAC) − ΔG_b°(KAC) = +1.33 kcal mol^{-1}. These results point to the hydrophobic nature of the COL binding site, which would render favorable nonspecific contacts with the nonpolar CH_2-CH_2CH_3 group. On the other hand, the NH_3^+ group in the same position on ring B would find itself in a thermodynamically unfavorable environment of low dielectric constant, which requires expenditure of extra free energy of ionization. The lower affinity of NAC could also reflect the electronic nature of the side group (*41*).

Inhibition of Microtubule Assembly. Inhibition of microtubule assembly is a post binding phenomenon. The control of inhibitory strength is a much more complicated phenomenon than binding. It does not follow the binding affinity, nor, on first inspection, is there any systematic dependence on structure. Let us examine in parallel the two sets of molecules. Taking the three ring compounds first, the inhibitory capacity (as measured by K_i) decreases weakly (by 0.27 kcal mol^{-1}) over the series KAC > COL = ALLO > MAC, all of which are strong inhibitors. For the two ring compounds, the order is TKB > MTC > TMB > TCB, with large steps of 0.82, 1.08, and >1.5 kcal mol^{-1} between the individual molecules in the series. Therefore, not only is the overall span much greater (>3.3 kcal mol^{-1}) than for the three ring compounds, but the order with respect to side group changes and the mode of inhibition spans the spectrum from strong substoichiometric to stoichiometric.

What is the essence of the role of ring B? For the system TCB → ALLO, it had been found that introduction of ring

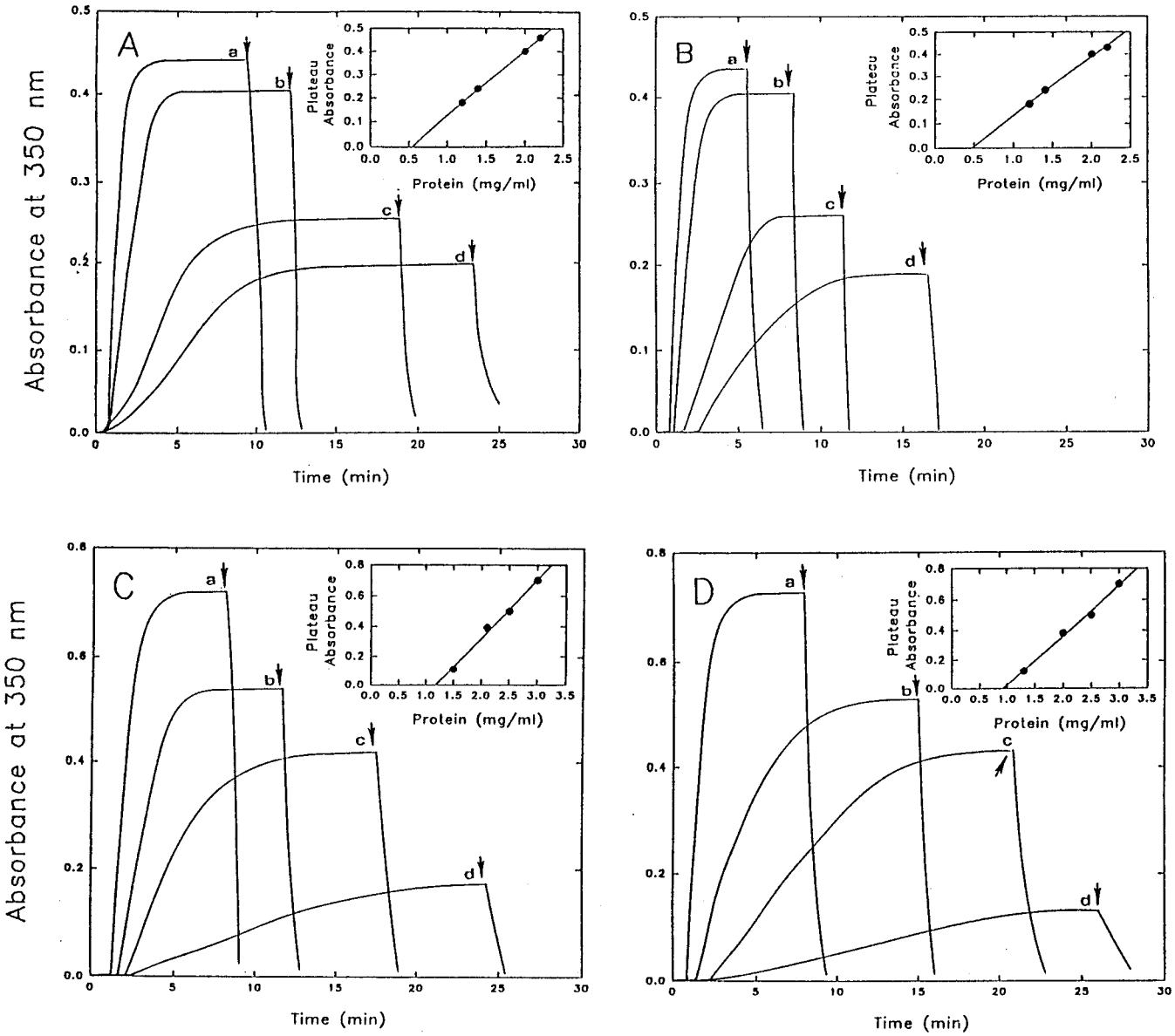


FIGURE 9: Turbidity time course of the abnormal polymerization induced by KAC, BAC, NAC, and MAC in 10 mM sodium phosphate buffer, pH 7.0, 16 mM $MgCl_2$, and 1 mM GTP. The concentration of drug was 8×10^{-5} M in each case. The arrows indicate cooling of the samples at 10 °C. (Panel A) Polymerization of tubulin induced by KAC when heated from 10 to 37 °C; the tubulin concentrations were (a−d) 2.3×10^{-5} M, 2.0×10^{-5} M, 1.43×10^{-5} M, and 1.3×10^{-5} M. (Panel B) Polymerization of tubulin induced by BAC when heated from 10 to 37 °C; the tubulin concentrations were (a−d) 2.2×10^{-5} M, 2.0×10^{-5} M, 1.4×10^{-5} M, and 1.2×10^{-5} M. (Panel C) Polymerization of tubulin induced by NAC when heated from 10 to 37 °C; the tubulin concentrations were (a−d) 3.0×10^{-5} M, 2.5×10^{-5} M, 2.1×10^{-5} M, and 1.5×10^{-5} M. (Panel D) Polymerization of tubulin induced by MAC when heated from 10 to 42 °C; the tubulin concentrations were (a−d) 3.0×10^{-5} M, 2.5×10^{-5} M, 2.0×10^{-5} M, and 1.3×10^{-5} M. (Insets) plateau absorbances as a function of total protein concentration.

B into the biphenyl compound strengthened inhibitory capacity by at least −3.4 kcal mol^{-1} (*11*). If the effects of groups were additive, as in binding, similar increments should be found in the other two pairs.[4] Our quantitative results show that, for the methoxy containing pair (TMB → MAC), ring B strengthened ΔG_i° by −1.77 kcal mol^{-1}. For the methyl ketone pair (TKB → KAC), the increment of inhibitory strength was only −0.15 kcal mol^{-1}. For comparison, COL was a stronger inhibitor than MTC by −0.85 kcal mol^{-1}. It is clear that there is no uniformity whatsoever in the quantitative contribution of ring B to the strength of inhibition. Hence, there is no additivity in inhibition. The very similar inhibitory strengths of the three ring compounds suggests that introduction of structural rigidity by ring B raises the inhibitory strength of all these compounds to a common maximal value. In this, ring B is a nonspecific passive entity which acts as a scaffolding that holds individual features of the drug molecule in proper orientation whether bound to the protein or not. Inhibition proceeds via the induction by the drug of a conformational change in the protein that is propagated allosterically to the loci of protein−protein contacts, which are formed in a geometry incompatible with microtubule assembly. The extent of that conformational change is determined by interactions between groups on the drug molecules and particular loci on the protein in the complex formed during binding. This can be

[4] In a screening type assay, MAC had been reported to be a strong inhibitor, but it had not been subjected to a quantitative scrutiny (*1*).

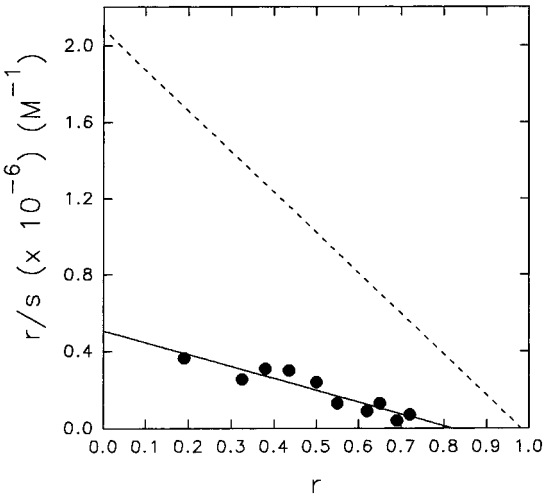


FIGURE 10: Scatchard plot of the titration of the GTPase activity induced by KAC shown as solid circles. The free ligand concentrations, *s*, were obtained from the total concentrations, protein concentration, and fractional enzyme activation, assuming a binding stoichiometry of 1. Protein concentrations were between 3×10^{-6} and 1×10^{-5} M; enzyme activity is expressed as moles of [^{32}P] phosphate released per mole of protein per minute, as described previously (*19*, *27*). The solid line is a least-squares fit of the experimental data [$K_b = (6.18 \pm 2.2) \times 10^5$ M^{-1}]. The dashed line is the least-squares fit of the experimental data of Figure 4A obtained by fluorescence titration, shown here for the purpose of comparison. *r* and *s* are the fraction of drug bound and free drug concentration, respectively. In the case of the GTPase titration, the fraction of maximal activity obtained was taken to be equal to the fraction of drug bound.

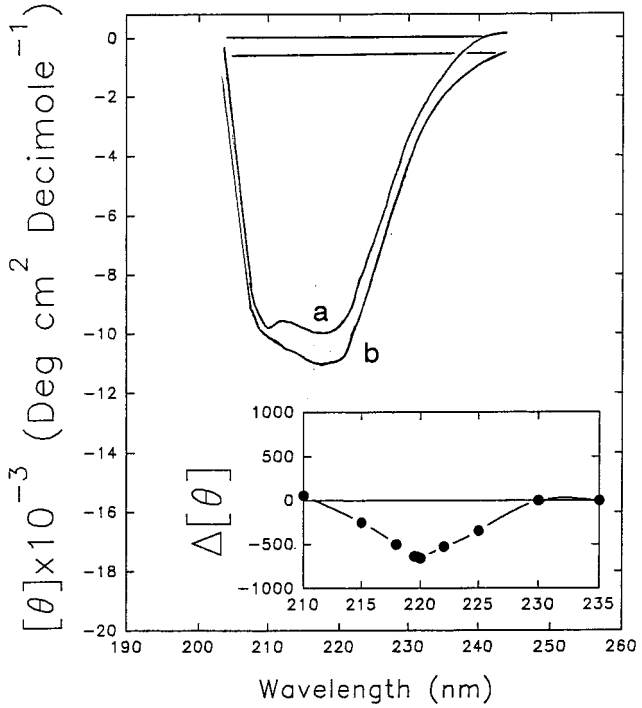


FIGURE 11: Effect of KAC on the circular dichroism spectrum of tubulin in 10 mM sodium phosphate buffer, pH 7.0, and 0.1 mM GTP at 20 °C: (a) spectrum of 2.0×10^{-5} M tubulin, (b) spectrum of 2.0×10^{-5} M tubulin, and 2.9×10^{-5} M KAC. Each spectrum is the average of duplicate samples. Tracing c is the result of subtracting tracing a from tracing b.

maximized by maintaining rigidly the appropriate drug moieties in optimal orientation within the complex. Ring B, by suppressing rotation about the biaryl bond, prevents wobbling of ring C′ of the complexed drugs and, thus, eliminates a source of destabilization of the induced protein conformation.

Since the three ring compounds inhibit microtubule assembly with essentially the same strength, the great difference in the increments of the standard free energy of inhibition when structural rigidity is introduced by ring B must reflect the difference between the abilities of the various two ring compounds to induce in the protein the necessary conformational change. In the case of the biphenyls, this difference must reside in the nature of the group attached at position 4′ of ring C′. In a previous study (*11*), it had been postulated that the induction of substoichiometric inhibition requires the COL family of molecules to penetrate within a pocket and be locked in a specific steric conformation that brings a ring C′ (or C) oxygen into proper contact with a key group in tubulin with the possible formation of a hydrogen bond or a π bond interaction (*11*, *34*, *42*, *43*). The binding of TKB seems to accomplish this well (*11*, *44*). Its carbonyl group finds itself in the spatial locus needed to enter optimally into interaction with the proper group(s) in the protein that triggers the linked protein conformational change needed to increase the binding strength of the complex to the growth end of a microtubule, ΔG_i°, over that of unliganded tubulin, ΔG_g°. For TKB, $\Delta G_i^\circ - \Delta G_g^\circ = -8.97 + 7.09 = -1.88$ kcal mol^{-1}; for KAC, it is -2.03 kcal mol^{-1}. Therefore, the presence of a ketone is sufficient to induce the full effect, and addition of ring B can make only a marginal contribution.

The other two biphenyls, TCB and TMB, are much weaker inhibitors than TKB. When transformed into the rigid three ring structures, ALLO and MAC, their binding locks the corresponding oxygens in the orientation needed to maximize the interaction with the key groups in the protein. TCB contains a carbonyl group in the same spacial locus as TKB (*44*). Its inability to act as a substoichiometric inhibitor has been ascribed to the steric interference of the bulky $COOCH_3$ group relative to the $COCH_3$ of TKB (*11*), which prevents it from penetrating sufficiently within the COL binding pocket (*44*) and interacting with the pertinent group on tubulin. This can be overcome by ALLO by virtue of its rigid three-ring structure at the loss of some binding free energy (weaker than that of KAC by 0.5 kcal mol^{-1}). This is consistent with the very low perturbation by TCB of the tubulin circular dichroism at 220 nm, while the binding of ALLO induces a perturbation identical with COL and the methyl ketones. The weak substoichiometric inhibition of microtubules by TMB indicates that the interactions of the methoxy group with particular groups in the protein locus are weaker than those of the ketone carbonyl. Both TMB and MAC (the three ring compound) bind more weakly to tubulin than TKB and KAC, by 0.50 and 0.84 kcal mol^{-1}, respectively. Sterically, both MAC and TMB can fit into the COL binding pocket as easily as KAC and TKB. Therefore, there is no steric interference to overcome and the difference with the ketone must reflect a difference in the nature of the interactions of the group in position 4′ with the binding pocket. For example, the keto compound would form a stronger hydrogen bond than the ether (*44*, *45*) and affect more strongly π bond formation. Furthermore, a low energy conformation of TKB can place its carbonyl in a locus 0.1 Å from that occupied in space by the COL carbonyl,

while for TMB the displacements of its oxygen from the loci of both the COL carbonyl and methoxy groups are 0.9 Å (*44*). The difference in the interactions between tubulin and the carbonyl and methoxy groups is supported by the fact that all carbonyl containing molecules induce the abnormal polymerization of tubulin at 37 °C, while the two methoxy molecules (MAC and TMB) require heating to a higher temperature (42 °C). It is noteworthy that, for this process, the introduction of ring B does not lower the temperature of abnormal polymerization induced by the methoxy compounds to that induced by the carbonyl-containing molecules. Abnormal polymerization is induced to similar extents by all the carbonyl-containing molecules, and ridigity of structure plays no role in the control of this process. Since this process requires binding saturation, it is a property of the tubulin−drug complex and is independent of the fluctuating interactions at low levels of binding at which microtubule assembly inhibition takes place.

Mechanism of the Inhibition. Mechanistically, inhibition proceeds through the binding of a tubulin−colchicine complex to the growth end of a microtubule (*12*). Since the loci of COL binding and interprotein contact(s) in complexation to a microtubule are distant from each other, the action must be propagated allosterically via a long-range conformational change in the protein induced by the binding. The simplest formal model description of the process is given by a set of three consecutive reactions (Scheme 3), namely binding (K_b), activation of the complex to the assembly inhibiting conformation (K^*), and binding of the active complex to the growth end of a microtubule (K'):

Scheme 3

$$T + C \rightleftharpoons TC;\ K_b$$

$$TC \rightleftharpoons T^*C;\ K^*$$

$$T^*C + M \rightleftharpoons MT^*C;\ K'$$

The observed inhibition constant, K_i, is, then, a measure of the two linked postbinding equilibria, $K_i = K^*K'$. As a heuristic probe of this simple model, one may make a simple calculation. On binding to tubulin, rings C (C′) of all the analogues are positioned within the same locus. Let us assume that, in the active state (T*C), all tubulin−drug complexes bind to the growth ends of microtubules in identical manner and with equal affinity, i.e., K' is the same for all the drugs of this series. The differences in the inhibitory capacities would then reflect the ability of each ligand to induce the conformational change in tubulin. Scheme 3, then, permits to calculate the fraction of liganded tubulin molecules present in the active protein conformation, T^*C. Let us take four molecules: a general strong inhibitor (an average between COL, ALLO, KAC, MAC, and TKB), MTC, TME, and TMB, and let us assign a strong affinity constant of the active complex (T*C) for the microtubule, $K' = 1 \times 10^6\ M^{-1}$, i.e., ten times the normal growth constant, K_g. Using the K_i values of Table 2, this gives values of K^* of 2.2, 0.56, 0.87, and 0.096 for the four drugs. Combination of these with the values of the parameter *r* of Table 2 shows that, at 50% inhibition, the fraction of liganded tubulin that is in the active conformation capable of inhibiting microtubules, expressed as percent of total tubulin−drug complex, is 69 for strong, 47 for TME, 36 for MTC, and 9 for TMB. This explains in a descriptive way the need of highly different concentrations of the drugs to induce the inhibition of microtubules which proceeds, in fact, by an identical chemical mechanism for all.

This mechanism can also account for stoichiometric inhibition. Let us take TCB for which K_i was immeasurable ($<10^4\ M^{-1}$). Table 2 shows that, at 50% inhibition, 40% of the tubulin was liganded. If we assign $K_i = 5 \times 10^3\ M^{-1}$, this gives $K^* = 0.005$, which leads to the result that only 0.5% of the formed tubulin−TCB complex is present in the active form that is capable of inhibiting microtubules, i.e., for each active complexes, 200 tubulin−TCB units are inactive. This leads to what appears as sequestration. Therefore, this simple mechanism can bring uniformity to the entire spectrum of inhibition, from strong substoichiometric to stoichiometric and removes the need of invoking two different mechanisms, namely (i) blocking of a microtubule end and (ii) sequestration. The mechanism of microtubule inhibition by the COL family of drugs is unique: it consists of the binding in the aberrant geometry of a tubulin−drug complex in the active conformation to the growth end of a microtubule (*12*, *13*). The difference between drugs resides in their ability to induce the active conformation after binding to the protein. The full mechanism must obviously contain much more detail. It must be emphasized that the present calculation is not pretended to be a rigorous analysis of the system. Its purpose is simply to illustrate the uniformity and basic simplicity of the binding and inhibitory processes for a set of molecules that, on first view, seem to behave in highly disparate fashion, and that only a detailed systematic study could discern. On a molecular level, the picture must contain a high degree of complexity, in the sense that details of contacts and motions of groups in the protein become known. This, however, will have to await the availability of the crystal structures of tubulin and of its complexes with the various drugs.

GTPase Activity. All the two or three ring analogues of COL were able to induce a GTPase activity in tubulin (see Table 3). The most striking result was that TME, which is ring C of COL, induces the GTPase activity. It is very weak, but reproducible and real. This means that ring C alone contains the controls necessary for inducing in tubulin the necessary alignment of the catalytic groups in the E-site of the enzyme which is at a distance of >2.4 nm from the COL binding site (*46*). Ring A by itself does not have this ability, since PODO does not induce the enzymatic activity in the protein. Therefore, ring C contains the necessary information for inducing both substoichiometric inhibition and GTPase activity. In the larger molecules, rings A and B serve as anchors; ring A contributes to the energy of binding, and ring B imparts structural rigidity in the proper orientation.

What is the specificity of the various groups on ring C (C′) in controlling the GTPase activity? It had been suggested previously that there is a direct relation between the standard free energy of ligand binding and the induction of the enzymatic activity (*7*, *19*). The availability of the four new three ring analogues shows that the situation may be more complex. The alignment of the drugs in descending order of the maximal rate of induced hydrolysis is (Table 3, column 2) COL > BAC = KAC > NAC > ALLO = TKB > MAC > MTC > TCB > TMB >> TME. This order

follows in general the binding affinities, with two exceptions: the methyl ketones NAC and TKB are displaced to higher GTPase activities than expected from their binding strengths.

GTPase Activity and Microtubule Inhibition: A Single Conformational Transition. Do these results mean that microtubule inhibition and GTPase induction proceed by way of the same conformational transition in the protein even though the orders of efficacy of the ligands are different for the two effects? Binding of the drugs leads to a conformational alteration of the protein. This alteration, however, may be directed differently to different sites on the protein molecule. The observation that the ketone enhances both activities suggests that the two are induced by the same general conformational change. Yet, GTPase activity, except for the ketones, follows the binding affinity, while inhibitory capacity deviates from it in a major way. Why this difference? The induction of the tubulin−COL GTPase activity is due to an allosteric effect (*19, 27, 38*) that involves two well-defined sites located in the same subunit (*31*), namely the COL binding site and the β subunit GTP binding site (E-site in tubulin). The transition is restricted to the alignment of the catalytic groups in the E-site of the enzyme in a favorable orientation for catalysis. This is favored by strong binding and some group specificity. Microtubule inhibition requires the strong binding of a tubulin−drug complex to the end of a growing microtubule (*11, 12*). This involves the formation of both longitudinal and lateral bonds with the growing polymer, i.e., the interaction of multiple sites on the $\alpha-\beta$ tubulin heterodimers, which may be distant from each other and, in fact, located on different subunits. The lateral bonds are highly susceptible to spatial perturbations in the mutual alignment of the protein subunits (*47*). Any difference in the end result of the propagation of the allosteric effect to the different interaction loci, i.e., different lateral bonds, would be observed as a difference in the extent of the pertinent conformational change, i.e., in the value of K^* of Scheme 3. This would be reflected in different values of the measured apparent binding constant to the microtubule end, K_i, although this difference would, in fact, reflect only the different efficacies of the drug molecules in inducing and propagating the necessary conformational change across the protein to the several noncontiguous loci active in the binding.

Conclusions. The present comparison of a series of two and three ring COL analogues that differ by the nature of their rings C′ (or C), has permitted to identify specific and nonspecific characteristics of the various manifestations of the interaction of these compounds with tubulin. These are the following.

(1) *Binding* is a noncooperative process. The energy of binding is provided by rings A and C which interact independently at specific subsites on tubulin. Ring A serves as anchor, and ring C contains the information needed to induce the specific consequences of binding. Ring B makes an entropic contribution by eliminating rotation about the A−C′ (or C) biaryl bond. Its introduction into four independent two ring compounds makes an identical contribution of -1.3 ± 0.2 kcal mol^{-1} of standard free energy. Its role, therefore, is to provide rigidity of structure in the proper orientation.

(2) The *immediate consequence of binding* is the induction in the protein of a conformational transition, which (i) causes the tubulin−drug complex to bind to the growth end of microtubules in a geometry incompatible with microtubule assembly, and (ii) induces a GTPase activity.

(3) The *GTPase activity* follows in general the binding affinity, with some enhancement by a methyl ketone in position 4′ of ring C′. Ring C alone contains the ability to induce the enzymatic activity, since ring A is inactive, while TME (ring C of COL) induces activity.

(4) *Inhibition of microtubule assembly* does not follow binding affinity, and there is no additivity of the contributions of structural moieties. Inhibitory capacity is modulated by the interactions of a ring C′(C) oxygen with groups in the protein. This requires the proper alignment of such groups within the COL binding cavity. It can be achieved by imparting rigidity to the structure by ring B, or by the introduction of a methyl ketone into position 4′ of ring C′ in the biphenyl compound. In the two ring series, the order of inhibitory strength is methyl ketone (TKB) > methyl ether (TMB) > > carbobenzoxy (TCB). Hence, the methyl ketone oxygen can enter into maximal interactions with key groups in the protein within the COL binding site; TCB cannot penetrate the locus, due to steric interference, supported by its very weak ability to perturb tubulin tryptophan CD at 220 nm; the oxygen of TMB interacts much more weakly than that of TKB, reflected also in its lesser ability to induce abnormal polymerization. Conversion of the last two compounds to the three ring analogues, ALLO and MAC, eliminates wobbling in the binding pocket and maintains the necessary contacts by structural rigidity. Microtubule inhibition is exercised through the allosteric propagation across the $\alpha-\beta$ tubulin heterodimer of a conformational change to a number of distant loci that must form longitudinal and lateral bonds in the binding to a growing microtubule. The difference in the inhibitory capacities of the drugs is due to their abilities to propagate this transition, i.e., to induce an active form of the tubulin−drug complex. This can explain in uniform manner the entire spectrum of inhibitions from strong substoichiometric to weak stoichiometric, which eliminates the need to invoke sequestration as the source of stoichiometric inhibition.

In light of these considerations it becomes clear that all controls reside in ring C (C′) and that only a methyl ketone in position 4′ of ring C′ of the biphenyl series (two and three ring) of analogues plays a highly specific role. It alone is sufficient to produce strong substoichiometric inhibition of microtubule assembly, which is only marginally increased by the introduction of ring B.

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