Stoichiometric and Substoichiometric Inhibition of Tubulin Self-Assembly by Colchicine Analogues[†]

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ABSTRACT: The mechanism of the stoichiometric and substoichiometric inhibitions of tubulin self-assembly by several structural analogues of colchicine (COL) was investigated. The inhibition data were analyzed in terms of a simple model that takes into consideration K_g, the normal microtubule growth constant, equal to Cr^{-1} (Cr is the critical concentration for microtubule formation), and K_b, the binding constant of the drug to tubulin. In this manner, the value of the microtubule inhibition constant (K_i), which is the binding constant of the tubulin−drug complex to the end of a growing microtubule (which stops the microtubule growth), was determined. The results of the analysis of microtubule inhibition by the various colchicine analogues show that all the inhibitions can be expressed reasonably by this model. The strongest inhibitors found were colchicine (COL), allocolchicine (ALLO), and the biphenyl keto analogue 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl (TKB), which had essentially identical values of $K_i = (2.1 \pm 0.3) \times 10^6$ M^{-1}. MTC, the two-ring analogue of colchicine, was weaker ($K_i = 5.6 \times 10^5$ M^{-1}). A most striking result was that tropolone methyl ether (TME), which is ring C of COL, and which binds very weakly to tubulin ($K_b = 3.5 \times 10^2$ M^{-1}), is a substoichiometric inhibitor. Its K_i value of 8.7×10^5 M^{-1} makes it identical in strength to MTC, suggesting that ring A makes little or no contribution to the induction of assembly inhibition. The three biphenyls, which bind to tubulin with similar affinity, spanned the spectrum from strong substoichiometric inhibition (TKB) to stoichiometric inhibition for 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB) and an intermediate mode for the methoxy derivative 2,3,4,4′-tetramethoxy-1,1′-biphenyl (TMB). The extent of tubulin bound to drugs at 50% inhibition (*r*) was ca. 2% for TKB, ALLO, and COL, i.e. one liganded tubulin for every 40−50 molecules of free protein (substoichiometric). This ratio was 1:1.5 for TCB (stoichiometric) and 1:6 for TMB (intermediate). For TME, which is a single ring compound, it was 1:25. The progression of the stoichiometries varied directly with K_i and was totally unrelated to the values of K_b, which indicated the control of the stoichiometry by K_i and the close thermodynamic linkage between *r* and K_i. Comparison of the inhibitory capabilities of the various drugs identified the need for strong substoichiometric inhibition of a carbonyl group on ring C or C′. Furthermore, this group must be properly oriented by interaction with the protein or by the structural rigidity imparted by ring B, as in ALLO. The simple linked equilibrium model developed in this paper permits the alignment of drugs along a continuum that ranges from stoichiometric to strong substoichiometric modes of microtubule inhibition. Furthermore, it shows that the previously identified two classes are the two ends of a monotonously progressing spectrum described by a single mechanism of action.

Tubulin is an $\alpha\beta$ heterodimer with a molecular weight in solution of 110 000 (Lee et al., 1973). Tubulin contains an exchangeable GTP[1]/GDP binding site in the β subunit (E site), which becomes nonexchangeable upon assembly into microtubules. GTP−tubulin is the active form of the protein. GDP−tubulin is inactive in microtubule assembly (Carlier & Pantaloni, 1978) and has been identified as the ground state of tubulin (Shearwin et al., 1994). The binding of colchicine and several structural analogues to tubulin induces a conformational change in the protein (Garland, 1978; Andreu & Timasheff, 1982b; Perez-Ramirez & Timasheff, 1994), which is manifested by the induction of assembly-independent GTPase activity (David-Pfeuty et al., 1977; Andreu & Timasheff, 1981; Perez-Ramirez et al., 1994), inhibition of microtubule formation (Andreu & Timasheff, 1982b; Medrano et al., 1989, 1991; Andreu et al., 1991), and self-assembly into structures other than microtubules (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983). These structures differ geometrically from microtubules in that they are sheets, ribbons, or amorphous aggregates, but their assembly pathway conforms

Chart 1

to nucleated polymerization, with thermodynamic characteristics similar to those of microtubule assembly (inhibition by Ca^{2+}, cold, requirement of GTP, similar variation of assembly standard free energy, enthalpy, entropy, and heat capacity changes). The alkaloid colchicine strongly inhibits microtubule assembly (Wilson & Bryan, 1974) at substoichiometric levels (Margolis & Wilson, 1977, 1978; Skoufias & Wilson, 1992), i.e. at small mole ratios of drug to tubulin. It is a three-ring structure (for structures see Chart 1) that consists of a trimethoxyphenyl ring (ring A) linked to a tropolone methyl ether (ring C) by a seven-membered ring (ring B). A detailed analysis of the binding process using single ring analogues of the trimethoxyphenyl and tropolone methyl ether parts of the colchicine molecule (Chart 1) has led to a simple thermodynamic model of the binding in terms of a bidentate mechanism; the tropolone methyl ether ring (ring C) and the trimethoxyphenyl ring (ring A) bind to two independent subsites on the protein (Andreu & Timasheff, 1982a). This thermodynamic model seems to describe the binding to tubulin of all the colchicine analogues studied (ALLO, TCB, TKB, TMB, and MTC) (Andreu et al., 1984, 1991; Medrano et al., 1989, 1991).

There are two mechanisms that might account for the substoichiometric poisoning of microtubule assembly by colchicine. (1) One is direct binding of the drug to free ends of microtubules. This would require tubulin molecules on the free end of a microtubule to have a much higher affinity for the drug than soluble tubulin, with the net consequence that microtubule ends could be liganded by colchicine, while unpolymerized αβ tubulin remained essentially unliganded. (2) The other is binding of the tubulin−colchicine complex to the free ends of microtubules, thereby blocking assembly in the proper geometry (Margolis & Wilson, 1977, 1978; Sternlicht & Ringel, 1979; Margolis et al., 1980; Lambeir & Engelborghs, 1980; Skoufias & Wilson, 1992). While

thermodynamically the two mechanisms are indistinguishable as they form two corners of a linkage box, kinetically, only one may be available. Skoufias and Wilson (1992) have shown that the second pathway is open.

Determination of the drug levels needed to inhibit microtubule formation has led to the conclusion that, while colchicine and some of the analogues inhibit microtubule formation substoichiometrically, others act stoichiometrically. With the aim of arriving at a rigorous quantitative definition of these two modes of inhibition, an equilibrium thermodynamic study of the inhibition by a series of colchicine analogues has been carried out. The results of these investigations are reported in this paper.

MATERIALS AND METHODS

Materials. Calf brains were obtained from a local slaughter house and transported on ice to the laboratory. GTP (sodium salt, type III) was purchased from Sigma. Glycerol was from Aldrich Chemical Co. Ultrapure ammonium sulfate and sucrose were obtained from ICN/Schwartz-Mann. Guanidine hydrochloride was from United States Biochemical (USB). Allocolchicine (ALLO), TKB, TCB, TMB, and TBO were synthesized as described before (Medrano et al., 1989, 1991). MTC was kindly provided by Dr. T. J. Fitzgerald (Florida State University, Tallahassee, FL). TME was obtained by methylation of tropolone as described previously (Andreu & Timasheff, 1982b). Other chemicals were of reagent grade.

Tubulin Preparation. Tubulin was prepared from fresh calf brains (1 h maximum after slaughter) by a modified Weisenberg procedure (Weisenberg et al., 1968; Na & Timasheff, 1980). Protein aliquots (40 mg, 40−50 mg/mL) were stored in liquid nitrogen in a buffer that consisted of 0.01 M sodium phosphate, 0.1 mM GTP, 0.5 mM MgCl$_2$, 1 M sucrose, and pH 7.0. Prior to each experiment, samples of tubulin were thawed at 20 °C, and the bulk of the sucrose was removed from the tubulin solution by a Sephadex G-25 dry column procedure (Na & Timasheff, 1980). The resulting protein solution was cleared of aggregates by centrifugation at 35000g for 30 min. The final equilibration of the protein with the desired buffer was by gel chromatography on a Sephadex G-25 column (Na & Timasheff, 1982). The protein was maintained on ice and used within 4 h of sucrose removal.[2] Tubulin concentrations were 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} (Na & Timasheff, 1981).

Assembly. The self-assembly of tubulin was monitored turbidimetrically (Gaskin et al., 1974; Lee & Timasheff, 1977) at 350−450 nm on a Cary 118 recording spectrophotometer. Tubulin equilibrated with PMG buffer (0.01 M sodium phosphate, 16 mM MgCl$_2$, 3.4 M glycerol, 1 mM GTP, and pH 7.0) was supplemented with the appropriate COL analogue and incubated at 20 °C for 30 min prior to assembly, if not otherwise indicated. The protein was then placed in a thermostatted cuvette maintained at 10 °C, and assembly was initiated by rapidly switching the water supply to a second water bath maintained at 37 °C. Assembly of tubulin has a typical critical concentration, Cr, of 1 mg/mL

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[2] When tubulin was kept longer than 4 h in the absence of sucrose, it was not possible to determine accurately the plateau absorbance attained upon assembly.

in PMG buffer. The degree of assembly in the presence of increasing concentrations of COL was also monitored by rapid centrifugation of assembled material. The results were in agreement with the turbidimetric analysis.

Ligand Concentrations. The concentrations of COL and its analogues were determined by ultraviolet absorption spectroscopy using the following extinction coefficients: COL, 15 950 M^{-1} cm^{-1} at 353 nm (Andreu & Timasheff, 1982a); ALLO, 11 860 M^{-1} cm^{-1} at 288 nm and 4680 M^{-1} cm^{-1} at 315 nm (Medrano et al., 1989); TBO, 15 900 M^{-1} cm^{-1} at 256 nm (Andreu et al., 1991); TKB and TMB, 14 400 and 16 130 M^{-1} cm^{-1} at 295 and 256 nm, respectively (Medrano et al., 1991); MTC, 17 600 M^{-1} cm^{-1} at 343 nm (Andreu et al., 1984); TCB, 12 100 M^{-1} cm^{-1} at 284 nm (Medrano et al., 1989); and TME, 25 900 M^{-1} cm^{-1} at 236 nm (Andreu & Timasheff, 1982b).

RESULTS

Effect of Colchicine Analogues on the in Vitro Microtubule Assembly. The interactions of ALLO, TCB, TKB, TMB, TBO, and MTC with tubulin have been characterized in previous studies (Andreu et al., 1984, 1991; Bane et al., 1984; Medrano et al., 1989, 1991; Hastie, 1989). All inhibit the binding of COL to tubulin, and the binding of each analogue is inhibited by COL and podophyllotoxin (Andreu et al., 1984, 1991; Medrano et al., 1989, 1991). All have been shown to inhibit the assembly of tubulin into microtubules, but at different levels. Therefore, it was deemed of interest to examine quantitatively the mechanisms by which these analogues perturb the in vitro microtubule assembly. The inhibitions of the self-assembly of tubulin into microtubules as a function of drug concentration are presented in Figures 1–3, and the results are summarized in Table 1. Quantitatively, the inhibitors seem to group themselves into three different classes. In the first, the molar concentration of drug needed to reduce the turbidity by 50% was of a magnitude similar to that of tubulin. This class contains TCB and TMB, while TBO is active at a lower drug to tubulin ratio. Examination of Figure 1A shows that the turbidity generated by the self-assembly of 2.6×10^{-5} M pure tubulin was reduced by half by 1.0×10^{-5} M TMB. Figure 1B shows the results for TBO which was needed at a concentration of 3.0×10^{-6} M to reduce by 50% the turbidity generated by the self-assembly of 2.2×10^{-5} M tubulin. Figure 1C shows that a similar reduction of turbidity generated by the self-assembly of 2.4×10^{-5} M tubulin required 1.9×10^{-5} M TCB. On the other hand, a second class of drugs, which consists of ALLO, MTC, and TKB, reduced the turbidity by 50% at concentrations 1–2 orders of magnitude lower than that of tubulin, as shown in Figure 2. The third class consists of the single ring analogues of COL, namely TME and NAM, for which 50% inhibition occurs at a 10–100-fold excess of drug. Figure 3 shows the results for TME, which is known to bind very weakly to tubulin. Nevertheless, it acts as a reasonably efficient inhibitor of assembly, the significance of which will be discussed later. The self-assembly of 2.3×10^{-5} and 2.6×10^{-5} M tubulin was reduced by 50% by 2.6×10^{-4} and 3.1×10^{-4} M TME, respectively, i.e. at concentrations 10 times higher than needed with TCB. Such a cursory examination of the results suggests that the three classes of drugs belong to stoichiometric, substoichiometric, and weakly stoichiometric inhibitors. Figure 4 shows that the extent of inhibition by all drugs
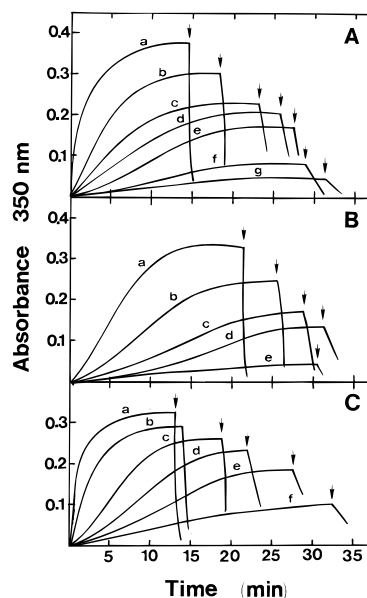

FIGURE 1: Effect of COL analogues on the turbidity time course of the in vitro microtubule assembly. The reaction was started by warming the solution in the assembly buffer [0.01 M sodium phosphate buffer (pH 7.0), 16 mM $MgCl_2$, 3.4 M glycerol, and 1 mM GTP] from 10 to 37 °C. Tubulin was preincubated with the analogues at 10 °C for 30 min prior to initiation of the assembly. (A) (a) 2.6×10^{-5} M tubulin; (b–g) same, with 4.9×10^{-6}, 6.0×10^{-6}, 8.9×10^{-6}, 1×10^{-5}, 1.5×10^{-5}, and 4.5×10^{-5} M TMB. (B) (a) 2.2×10^{-5} M tubulin; (b–e) same, with 1.3×10^{-6}, 3.0×10^{-6}, 3.5×10^{-6}, and 8.7×10^{-6} M TBO. (C) (a) 2.4×10^{-5} M tubulin; (b–f) same, with 4.9×10^{-6}, 9.3×10^{-6}, 1.1×10^{-5}, 1.4×10^{-5}, and 2.2×10^{-5} M TCB. The critical concentration of microtubule assembly under these conditions was 1.0 ± 0.1 mg/mL tubulin. The arrows indicate cooling of the samples to 10 °C. In all cases, the turbidity decreased to the original value; the lines on the figure were not drawn fully to avoid crowding.
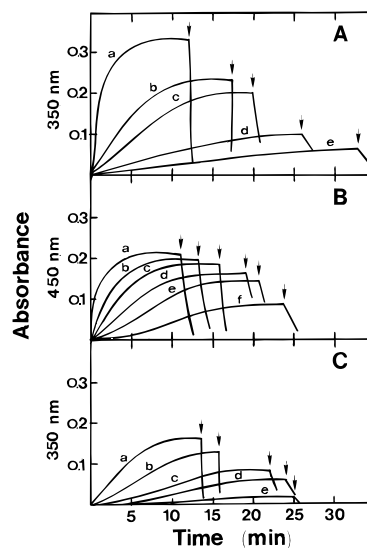

FIGURE 2: Effects of ALLO, MTC, and TKB on the turbidity time course of the in vitro microtubule assembly. Conditions were the same as in Figure 1. (A) (a) 2.4×10^{-5} M tubulin; (b–e) same, with 3.1×10^{-7}, 4.0×10^{-7}, 7.1×10^{-7}, and 1.8×10^{-6} M ALLO. (B) (a) 2.5×10^{-5} M tubulin; (b–f) same, with 1.2×10^{-7}, 4.5×10^{-7}, 7×10^{-7}, 1.1×10^{-6}, and 3.1×10^{-6} M MTC. (C) (a) 1.6×10^{-5} M tubulin; (b–e) same, with 2.1×10^{-7}, 4.8×10^{-7}, 6.6×10^{-7}, and 3.6×10^{-6} M TKB. The arrows indicate cooling of the sample to 10 °C. All assemblies reversed fully.

increased monotonously with the mole ratio of total ligand to total protein in the solution. As seen in Figure 4A, for

Table 1: Inhibition of Tubulin Self-Assembly by Colchicine Analogues

			typical single experiment				
analogue	K_b (M^{-1})	protein concentration range (M)	tubulin (M)	drug concn at 50% inhibition (M)	K_i (M^{-1})	ΔG_i° (kcal mol^{-1})	r^a (%)
COL	1.6×10^7 (37 °C)[b]	$1.7-2.2 \times 10^{-5}$ (3)[h]	2.2×10^{-5}	4.2×10^{-7}	$(2.2 \pm 0.2) \times 10^6$	-9.00	1.9 ± 0.2
ALLO	9.0×10^5 (37 °C)[c]	$1.6-2.4 \times 10^{-5}$ (3)	2.4×10^{-5}	5.0×10^{-7}	$(2.1 \pm 0.5) \times 10^6$	-8.97	1.9 ± 0.46
TKB	1.9×10^5 (25 °C)[d]	$1.6-3.0 \times 10^{-5}$ (5)	2.1×10^{-5}	8.5×10^{-7}	$(2.1 \pm 0.31) \times 10^6$	-8.97	2.2 ± 0.4
TCB	1.0×10^5 (25 °C)[c]	$2.0-2.4 \times 10^{-5}$ (5)	2.4×10^{-5}	1.9×10^{-5}	$<10^4$	>-5.6	40 ± 9
TMB	8.2×10^4 (25 °C)[d]	$1.8-2.6 \times 10^{-5}$ (5)	2.6×10^{-5}	9.8×10^{-6}	$(9.6 \pm 1.34) \times 10^4$	-7.07	17 ± 2
MTC	3.6×10^5 (37 °C)[e]	$1.8-3.0 \times 10^{-5}$ (3)	2.5×10^{-5}	1.8×10^{-6}	$(5.6 \pm 0.2) \times 10^5$	-8.15	5.8 ± 0.2
TBO	1.8×10^5 (25 °C)[f]	2.2×10^{-5} (3)	2.2×10^{-5}	3.0×10^{-6}	$(3.0 \pm 0.33) \times 10^5$	-7.77	8.1 ± 1.2
NAM	3.0×10^2 (37 °C)[g]	1.7×10^{-5} (1)	$1.7 \times 10^{-5\,g}$	$2.6 \times 10^{-3\,g}$	$<10^3$	>-4.2	46
TME	3.5×10^2 (37 °C)[g]	$2.3-2.6 \times 10^{-5}$ (3)	2.6×10^{-5}	3.1×10^{-4}	$(8.7 \pm 0.4) \times 10^5$	-8.42	4.2 ± 0.8

[a] Liganded tubulin at 50 % turbidity. The $\pm$ values reflect the spread between individual experiments; they do not take into account the uncertainties of the K_b values. [b] Calculated from the data of Diaz and Andreu (1991). [c] Medrano et al. (1989) and Shearwin and Timasheff (1994). [d] Medrano et al. (1991). [e] Andreu et al. (1984). [f] Andreu et al. (1991). [g] Andreu and Timasheff (1982b). [h] Number of independent experiments.
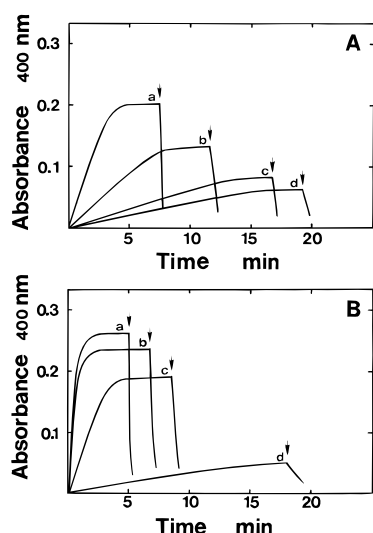

FIGURE 3: Inhibition of tubulin self-assembly by TME. (A) (a) 2.3×10^{-5} M tubulin; (b–d) same, with 2.1×10^{-4}, 3×10^{-4}, and 7.9×10^{-4} M TME. (B) (a) 2.6×10^{-5} M tubulin; (b–d) same, with 7.3×10^{-5}, 1.3×10^{-4}, and 8.3×10^{-4} M TME. Tubulin was preincubated with TME at 20 °C for 90 min before initiation of the self-assembly reaction. Assembly was carried out by heating the samples from 10 to 37 °C. The arrows indicate cooling of the samples at 10 °C, which fully reversed all assemblies.

TCB, 50% inhibition occurred at a mole ratio of 0.61 mol of drug per mole of tubulin. For TMB, this ratio was 0.38, and for TBO, it was 0.14. This suggests a stoichiometric to weakly substoichiometric mode of inhibition. For COL, ALLO, and TKB (Figure 4B), 50% inhibition occurred at mole ratios of 0.019, 0.021, and 0.031, respectively, which indicates substoichiometric inhibition. For MTC, the two-ring analogue of COL, 50% inhibition of tubulin self-assembly was obtained at a mole ratio of 0.07 (Figure 4B), again suggesting a substoichiometric mode. The inhibition results presented in Figure 5 in the form of isotherms as a function of free analogue concentration show the repeatability of the measurements, as points from various individual experiments performed at somewhat different protein concentrations overlap randomly. The lack of any significant divergence with protein concentration is consistent with previous observations that the binding of the ligands is independent of protein concentration and does not induce any changes in the sedimentation pattern of tubulin (Andreu et al., 1984; Medrano et al., 1989). Inhibition, however, is kinetically linked to binding, and the binding affinities of these various ligands differ by 1–2 orders of magnitude.

Therefore, classification of any particular drug as stoichiometric or substoichiometric simply from the level of drug at which inhibition occurs may be precarious. Such classification, in fact, requires that the linkages and competitions involved in the binding and microtubule arrest processes be taken into account and expressed in quantitative manner.

Stoichiometric and Substoichiometric Inhibitions. The question of stoichiometric versus substoichiometric inhibition was addressed in a quantitative manner by using the microtubule inhibition data obtained for the various drug: tubulin concentration ratios. It has been accepted generally that stoichiometric inhibition means sequestration of tubulin into a complex with the drug, which is incapable of binding to growing microtubules, while substoichiometric inhibition means the blocking of microtubule growth by the binding either of a tubulin drug complex or of a drug molecule to the growing polymer (Margolis & Wilson, 1977, 1978; Skoufias & Wilson, 1992). For inhibition by COL, Skoufias and Wilson (1992) have shown the blocking entity to be the stable tubulin–COL complex. The simplest mechanism that can describe quantitatively the inhibition process, whether by sequestration of the protein by complexing with the drug or by binding of the tubulin–drug complex to the end of a microtubule, is given by reaction scheme 1:

$$M_{n-1}TA \xrightleftharpoons{K_i} TA + M_{n-1} \xrightleftharpoons{K_b} A + T + M_{n-1} \xrightleftharpoons{K_g} M \quad (1)$$
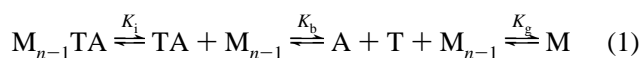
where K_g is the normal microtubule growth constant, equal to Cr^{-1} (Cr is the critical concentration in the absence of drugs); K_b is the binding constant of the drug to tubulin; K_i is the microtubule inhibition constant, which is the binding constant of the tubulin–drug complex to the end of a growing microtubule, the consequence of which is the stopping of microtubule assembly; 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 this simple mechanism, the assumption is made that the binding of one tubulin–drug complex to the end of a microtubule stops the growth of that polymer.[3] Arrest of

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[3] Inhibition by direct binding of a drug molecule to the end of a microtubule is simply described by the combination of the two steps on the left of the reaction scheme and their competition with microtubule growth by one additional tubulin protomer. Since M = M_{n-1}T, this gives $K_i' = K_bK_i/K_g$, where K_i' is the assembly-arresting effective binding constant for the equilibrium M + A $\rightleftharpoons$ MA. In the tubulin–COL complex, this pathway seems to be blocked kinetically (Skoufias & Wilson, 1992).
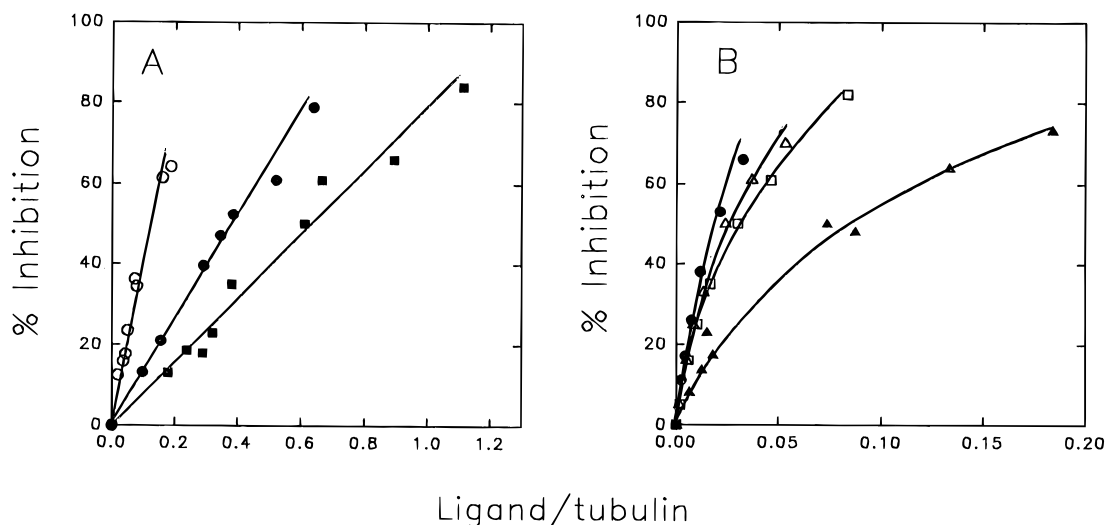
FIGURE 4: Fraction of the reduction of the plateau absorbance values (inhibition) as a function of the ratio of total ligand concentration to total protein concentration. (A) TMB (●), TBO (○), and TCB (■). (B) COL (●), ALLO (△), TKB (□), and MTC (▲).

growth by sequestration is described simply by setting $K_i = 0$; i.e. the binding of the complex TA to a microtubule is so weak that it does not occur at measurable concentrations. The model expressed by eq 1, in fact, consists of two competing reactions: (1) elongation of a microtubule by T ($K_g = Cr^{-1}$) and (2) binding of the drug to T → TA (K_b), followed by addition of TA to a growing microtubule (K_i).

Since the inhibition was measured by the decrease of turbidity induced by the addition of drugs, an inhibition equation was derived which related the decrease in turbidity to the equilibrium of eq 1. The derivation of the inhibition equation is given in the Appendix. It is based on two premises: (1) the well-established fact that turbidity is proportional to the mass of tubulin assembled into microtubules and (2) the assumption that only microtubules generate turbidity, while the blocked species, $M_{n-1}TA$, do not. The basis for this assumption is that, at steady state, the inhibited species $M_{n-1}TA$ are expected to be short.[4] As shown in Table 1, at 50% inhibition, 2% of the tubulin is liganded even for the strongest inhibitors. The strictly statistical chance of a TA kinetic unit becoming attached to a microtubule is, therefore, 1 in 50. Such attachment, however, stops microtubule growth, which, as a consequence, would occur at a low degree of polymerization. More rigorously, the probability is proportional to K_i/K_g. Since $K_g = 1 \times 10^5 M^{-1}$, a value of K_i of $1 \times 10^6 M^{-1}$, which is characteristic of the strong inhibitors as will be shown below, should render even higher the probability of a growth-blocking TA unit becoming attached to the growing microtubule. Microtubules being a dynamic state, blocked polymers disassemble at the other end and do not become longer. In terms of this analysis, the fraction of the turbidity

measured in the presence of an inhibitor relative to that in the absence of inhibitor is found to be (see Appendix)

$$\text{fraction} = \frac{1}{1 + K_b K_i K_g^{-1}[A]} - \frac{K_b K_g^{-1}[A]}{(T_{total} - Cr)(1 + K_b K_i K_g^{-1}[A])} \quad (2)$$

The data on the inhibition of microtubule assembly by the various drugs, shown in Figures 1−3, were analyzed in terms of eq 2, by solving simultaneously the points for all the drug−tubulin proportions used in any one experiment and averaging the K_i values obtained. The results are presented in Table 1.[5] The strongest inhibitors were found to be COL, ALLO, and TKB, with apparent standard free energy changes for the binding of the complex to a microtubule end (at 37 °C) of −9.00, −8.97, and −8.97 kcal mol⁻¹, respectively. For MTC, the value was lower, −8.15 kcal mol⁻¹. For TMB, however, the standard free energy of inhibition was considerably lower, −7.07 kcal mol⁻¹, i.e. a value very similar to the standard free energy of binding of the drug to tubulin. For TCB, the values varied between weakly positive and negative, indicating at best very weak binding of the complex to the end of a microtubule. A particularly interesting result was that obtained with TME, which binds to tubulin very weakly. It displayed an inhibition constant 3 orders of magnitude greater than its binding constant, and similar to that for MTC, but considerably higher than K_i for TMB.

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[4] The law of light scattering changes for shorter structures in such a manner that the scattering capacity per unit mass becomes progressively smaller than that of normal microtubules for assemblies with length L, smaller than the wavelength of the light, λ, as the dependence of tubidity changes from $(L/\lambda)^{-3}$ to $(L/\lambda)^{-4}$ (Berne, 1974; Timasheff, 1981; Andreu & Timasheff, 1986). Since in the present studies the wavelength used was 350−450 nm, the rapid decrease in scattering capacity will occur for microtubules that contained <600−750 $\alpha\beta$ tubulin dimers. Assemblies or aggregates of 200 $\alpha\beta$ tubulin dimers or less would generate scattering negligibly small (≤10%) per unit mass relative to long microtubules.

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[5] The inhibition data were obtained at 37 °C. Whenever available, the binding constants used were those measured at 37 °C. Otherwise, K_b values measured at 25 °C were used. For those molecules for which K_b is available at both temperatures, the differences between the two values are small. As a control, K_i was varied in eq 2 by the difference between the 25 and 37 °C values measured for MTC which has a strong temperature dependence (Andreu et al., 1984). The uncertainty introduced in $\Delta G_i°$ was 0.5 kcal mol⁻¹. The effect on r was ±0.2, i.e. within the experimental error. Furthermore, it should be noted that assembly was performed in a buffer that contained glycerol and Mg²⁺, while binding was measured in the absence of glycerol. It is known, however, that the binding equilibrium constants are identical in the two-solvent systems (Medrano et al., 1989; Perez-Ramirez & Timasheff, 1994).
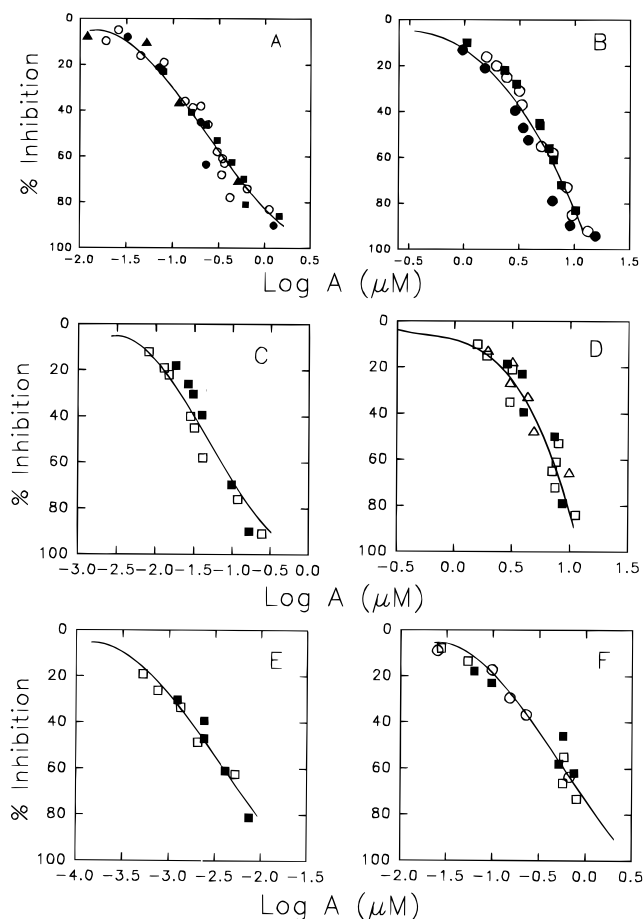
FIGURE 5: Effect of tubulin concentration on the reduction of the plateau absorbance values (inhibition) as a function of free drug concentration ([A]), plotted as isotherms. (A) Effect of TKB; ■, ▲, ○, and ●: 3.0×10^{-5}, 2.23×10^{-5}, 2.1×10^{-5}, and 1.6×10^{-5} M tubulin, respectively. (B) Effect of TMB; ■, ○, and ●: 2.6×10^{-5}, 2.2×10^{-5}, and 1.8×10^{-5} M tubulin, respectively. (C) Effect of ALLO; ■ and □: 2.4×10^{-5} and 1.6×10^{-5} M tubulin, respectively. (D) Effect of TCB; ■, □, and △: 2.4×10^{-5}, 2.0×10^{-5}, and 2.2×10^{-5} M tubulin, respectively. (E) Effect of COL; ■ and □: 2.2×10^{-5} and 1.7×10^{-5} M tubulin, respectively. (F) Effect of MTC; ■, ○, and □: 3.0×10^{-5}, 2.5×10^{-5}, and 2.0×10^{-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. The solid lines are the theoretical curves obtained by simultaneous fitting of the experimental values of K_b, K_i, and protein concentrations, at different inhibition fractions, to eq 2. In the case of TCB, the fitting was done using eq A7. 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: TKB, $K_b = 1.9 \times 10^5$ M^{-1}, $K_i = 2.1 \times 10^6$ M^{-1}, $T = 2.23 \times 10^{-5}$ M; TMB, $K_b = 8.2 \times 10^4$ M^{-1}, $K_i = 9.6 \times 10^4$ M^{-1}, $T = 2.20 \times 10^{-5}$ M; ALLO, $K_b = 9.0 \times 10^5$ M^{-1}, $K_i = 2.1 \times 10^6$ M^{-1}, $T = 2.0 \times 10^{-5}$ M; TCB, $K_b = 1.0 \times 10^5$ M^{-1}, $K_i = 0$, $T = 2.20 \times 10^{-5}$ M; COL, $K_b = 1.6 \times 10^7$ M^{-1}, $K_i = 2.2 \times 10^6$ M^{-1}, $T = 1.95 \times 10^{-5}$ M; MTC, $K_b = 3.6 \times 10^5$ M^{-1}, $K_i = 5.6 \times 10^5$ M^{-1}, $T = 2.50 \times 10^{-5}$ M.

The inhibition results were also examined in an alternate way by calculation of the extent of liganding of tubulin at 50% reduction of turbidity, r, directly from the binding constant, K_b, and the requirement that the equilibrium concentration of unliganded unassembled tubulin be equal to the critical concentration for assembly.[6] Thus, while ΔG_i° gives the inhibitory potency of any drug molecule, r gives the corresponding stoichiometry of binding. The two

parameters are necessarily linked in the thermodynamic sense. The results are listed in column 8 of Table 1. The general observations are as follows. (1) There is no clear division of the inhibitors into two distinct classes, but rather, the pattern falls into a spectrum from very low ligand binding at 50% inhibition to close to half of the tubulin being liganded, with some intermediate values. (2) There is no correlation of r with the binding constant, K_b, even though r is calculated from K_b, but a good correlation with the inhibition constant, K_i, as the two parameters vary inversely with each other. This testifies to the linkage between them. By this criterion also, it is very clear that COL, ALLO, and TKB are essentially equally strong substoichiometric inhibitors with ca. 2% of tubulin liganded at 50% reduction of turbidity, while MTC is somewhat weaker. The three biphenyls, TKB, TMB, and TCB, although very similar structurally, span the full spectrum of the inhibition pattern. The ketone, TKB, is a strong substoichiometric inhibitor. At 50% inhibition, for every liganded tubulin molecule, 45 $\pm$ 7 tubulins remain free. The ester, TCB, however, is at the other extreme of the spectrum. Its inhibition constant is indistinguishable from 0, and the data can be described by eq A7 (see Appendix). At 50% inhibition, only ca. 1.5 tubulins remain unliganded for every molecule that has bound TCB. The methoxy analogue, TMB, is intermediate. Its ratio of unliganded to liganded tubulins at 50% inhibition is ca. 5.0. A most striking result is that TME, the ring C analogue of COL, which binds with an affinity of $K_b = 3.5 \times 10^2$ M^{-1}, is, in fact, a substoichiometric inhibitor, since, at 50% inhibition of turbidity, for every molecule of tubulin liganded, ca. 25 remain free. The same is not true of the ring A analogue, NAM, which inhibits stoichiometrically. Its value of K_i, in fact, is close to 0, and its inhibition can be described by eq A7. Therefore, individually, the two moieties of COL do not form a common class of inhibitors, as could have been naively concluded from simple examination of the raw data, i.e. the molar ratio of total drug added to protein needed to reduce turbidity by 50%. From these results, it is clear that the mode of inhibition of microtubules by the COL family of drugs is determined not by the tubulin-liganding constant, K_b, but by the binding of the complex to the end of a microtubule, K_i. Looking at the substoichiometric inhibitors, for ALLO and TKB, $K_i > K_b$ by 1 order of magnitude, while for COL, K_i is 1 order of magnitude smaller than K_b. For TME, K_i is more than 3 orders of magnitude greater than K_b. Therefore, binding alone cannot lead to an unequivocal determination of the mode of inhibition. This is given by K_i.

DISCUSSION

Model of the Inhibition Mechanism. In previous studies, the mechanisms of COL binding to tubulin and the induction of the GTPase activity in the complex had been characterized extensively by the use of simple structural analogues of colchicine (Andreu et al., 1982a,b, 1984, 1991; Medrano et al., 1989, 1991; Timasheff et al., 1991; Perez-Ramirez et al., 1994; Perez-Ramirez & Timasheff, 1994). The present

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[6] In this calculation, the binding to microtubule ends can be neglected since their molar concentration is some 4 orders of magnitude lower than that of $\alpha\beta$ protomeric tubulin. Occasional trapping of a drug−tubulin complex into the polymer would also make no significant contribution for the same reason.

study has scrutinized the effect of these same analogues on the inhibition of tubulin self-assembly into microtubules. Analysis of the inhibition data was carried out in terms of a simple competition equilibrium between the growth of microtubules and their arrest by the binding of a single tubulin−drug complex to a growing microtubule (eq 1). The simplicity of this model permits the characterization of the inhibitory capacity of any drug in terms of an inhibition constant, K_i, from turbidity data that had been properly corrected for nonassembling protein (Monasterio & Timasheff, 1987). This, in turn, permits the comparison of inhibitors in a way that is systematic and independent of the total drug to protein concentration ratio. The analysis of the inhibition data in terms of this equilibrium model is independent of the pathway followed by the drug to bind to microtubules, whether directly or complexed to tubulin, since the two kinetic pathways are thermodynamically linked through a proper box. For each drug, the inhibition curve was described well by a single value of K_i.

The model adopted in these studies is the simplest one that can describe the inhibition process. Examination of Figure 5 shows that the theoretical curves calculated with eq 2 and the values of K_i and K_b listed in Table 1 give a reasonable fit of the experimental data for all the inhibitors. The slight deviations may be attributed to experimental error in measuring the extent of inhibition at the two ends of the isotherm, as well as to the necessarily approximate nature of the model. The microtubule assembly−disassembly process is known to be kinetically complex. Even at steady state in a large excess of GTP, the assembled structures are in a dynamic state, marked by events such as spontaneous depolymerization, growth, annealing, breaking, etc. Furthermore, inhibition by drugs can involve kinetically the binding of more than one drug molecule to a microtubule, and conceivably a break within the structure, although such a case has not been reported. Furthermore, it has also not been excluded that additional tubulin molecules could add to the ends of protofilaments that are free of drug until cessation of growth. The present model, evidently, does not take specifically these various kinetic phenomena into consideration. It must be regarded, therefore, as the simplest mode of describing what is occurring at a pseudo-equilibrium, and the mass balance used in the analysis must also be regarded as an instantaneous view of the system, or a time average. The inhibition constant, K_i, is then an effective parameter that gives a quantitative measure of the ability of a compound to inhibit microtubule assembly, which, in turn, permits the comparison in a rational manner of the inhibitory action of related molecules. The frequently used approach of the simple observation of the molar ratio of total drug to tubulin at which turbidity is decreased by 50% can result in the shielding of the true situation, as, for example, is the case with TME which would be identified as a stoichiometric inhibitor by such an approach, since 50% inhibition occurs at a drug to tubulin ratio of 12. The simplicity of the current equilibrium model of the inhibition does find support, however, for the COL family of molecules in the studies of Skoufias and Wilson (1992), who have shown that COL inhibition is induced by the binding of a single tubulin−COL complex to the end of a growing microtubule, while free COL is unable to bind to that site. Furthermore, simulation of microtubule dynamics has shown that a single tubulin−drug complex bound to the microtubule lattice

suppresses growth (Bayley et al., 1994). Finally, the value of K_i for COL agrees with that previously reported for the binding of a tubulin−COL complex to a microtubule (Lambeir & Engelborghs, 1980).

What Makes an Inhibitor Substoichiometric? COL and ALLO are three-ring structures with different C rings (see Chart 1). The structures of MTC and TCB are derived from the first two by excision of ring B. This leads to a weakening of the binding by identical amounts ($\Delta G°$ increases by 1.4 ± 0.1 kcal mol^{-1}) whether ring C is a tropolone methyl ether (COL) or a carbomethoxybenzene (ALLO) (Medrano et al., 1989; Timasheff et al., 1991). The same is not true for microtubule inhibition as expressed through the inhibition constant K_i. The COL → MTC transformation increases the standard free energy of inhibition by 0.8 kcal mol^{-1}, while the ALLO → TCB transformation dramatically weakens the inhibition to the extent that K_i cannot be measured for TCB (see Table 1) and the mode of inhibition is changed from substoichiometric to stoichiometric. The fact that MTC remains substoichiometric, just as is COL, shows that substoichiometric inhibition does not require a three-ring structure.

Examination of the inhibitory capacity of the various analogues, shown in Table 1, reveals that TKB, which is a biphenyl analogue of ALLO in which the COOCH$_3$ group in position 4′ has been replaced by COCH$_3$, is as strong an inhibitor as COL. Yet, its binding constant[5] to tubulin is 80 times smaller than that of COL. This striking result proves that weaker binding does not need to lead to poorer inhibition capacity. This means that the two processes are not closely thermodynamically linked. In fact, TME, which has the weakest binding constant to tubulin (3.5×10^2 M^{-1} at 37 °C), has an inhibition constant more than 2 orders of magnitude greater (8.7×10^5 M^{-1}) and is intrinsically a substoichiometric inhibitor. The strong inhibitory capacity of TKB is in contrast to that of the other two biphenyl analogues. TCB is a stoichiometric inhibitor, while TMB is an intermediate case with an inhibitory free energy 1.9 kcal mol^{-1} weaker than that of TKB. Yet the binding free energies of the three compounds to tubulin are very similar. What is the source of this difference? Since the only difference between the three molecules is the group in position 4′ of ring C′, the necessary conclusion is that the methyl ketone group in that position has the ability to enter into those interactions with the protein that are required for induction of substoichiometric inhibition, while a carbobenzoxy group does not have that ability and a methoxy group has it at best weakly. Furthermore, the fact that TME is a substoichiometric inhibitor shows that the COL ring C alone is sufficient to bring on this effect. Its attachment to ring A with the formation of MTC has no significant effect on the strength of the inhibitory capacity. On the other hand, the two-ring analogue, TKB, in which COL ring A is attached to a methyl ketone-containing phenyl ring (ring C′) has a K_i value almost four times that of MTC. How is this strong inhibition induced?

The inhibition of microtubule growth by the drugs requires the induction of an altered conformation in tubulin subsequent to the binding of the drug (Andreu et al., 1983). This must be generated by the liganding of the drug to tubulin in a final state of proper orientation and complexation of

structural elements of the drug to elements of the protein structure. The binding of ring C (or C′) to tubulin involves a stacking interaction (Andreu & Timasheff, 1982a,b; Rava et al., 1987; Hastie, 1989; Hastie & Rava, 1989). It has been suggested that the tubulin−ring C (or C′) interaction also involves the formation of a hydrogen bond (Andreu & Timasheff, 1982a,b). Ring C of COL contains both keto and methoxy groups. What is the role of each in the induction of substoichiometric inhibition? As shown with TMB, the methoxy group by itself does not have the capability to induce strong substoichiometric inhibition. TMB binds to tubulin with an affinity similar to that of TKB, yet its inhibiting power is 1.9 kcal mol^{-1} weaker. TKB, which is the strongest inhibitor, contains only a carbonyl group. Therefore, the strong inhibiting power must reside in the presence of the carbonyl group in the proper position and orientation. ALLO and TCB also possess such a group in an identical position as part of the methyl ester. Yet, TCB is not capable of inhibiting substoichiometrically. This was a surprising result. At the outset of these studies, it was expected that TCB would be the strongest inhibitor among the biphenyls, since it is structurally the closest to the strong substoichiometric inhibitor, ALLO. What is the source of this puzzle? One possible answer is the difference in lengths between the COOCH$_3$ group and the COCH$_3$ group of TKB. The longer group in position 4′ of TCB, through its bulk, might prevent it from sterically entering into the interactions required for substoichiometric inhibition. The puzzle becomes then why is ALLO, which has the same group in position 4′, a substoichiometric inhibitor, since its binding would be subjected to the same steric hindrance? The answer may be found in the rigid structure of ALLO which could hold the position 4′ carbonyl in the proper orientation for the required interactions to occur. The extra free energy gained from the absence of the inter-ring free rotation found in TCB could compensate for the steric strain required in the formation of the contact between the COOCH$_3$ carbonyl and the proper locus on tubulin. Such steric strain should not exist in TKB by virtue of the lower bulk of the COCH$_3$ group. Hence, TKB can act as a very strong substoichiometric inhibitor of microtubule formation. To test these hypotheses, the structures of the various analogues have been determined by X-ray diffraction and compared with that of COL. The results are described in the following paper (Rossi et al., 1996).

Finally, one might ask the following question: what inter-tubulin interaction does the tubulin−drug complex affect? While lack of knowledge of the three-dimensional structure of tubulin precludes a detailed analysis of the effects of COL and analogues on its mode of assembly, it seems plausible 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 assembly undergone by tubulin (Timasheff, 1991). The lateral bonds, however, are highly susceptible to spatial perturbations in the mutual alignment of the protein subunits (Melki et al., 1989). The slightly different conformational states of tubulin induced by the various ligands may manifest themselves thermodynamically as a weakening of the lateral bonds at the end of a microtubule. This should prohibit the next tubulin molecule from forming the proper lateral bonds within a microtubule. As a consequence, growth should stop.
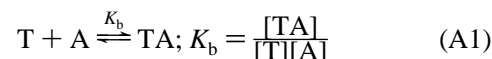
APPENDIX

Derivation of the Inhibition Equation. Consideration of the inhibition reaction scheme (eq 1) leads to the following equilibria.
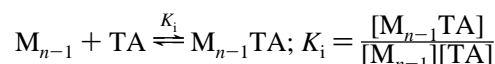
(1) Microtubule growth:

$$M_{n-1} + T \overset{K_g}{\rightleftharpoons} M; \quad K_g = \frac{[M]}{[T][M_{n-1}]}$$
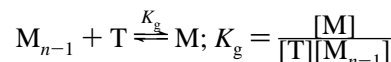
(2) Binding of drug to tubulin:

$$T + A \overset{K_b}{\rightleftharpoons} TA; \quad K_b = \frac{[TA]}{[T][A]} \tag{A1}$$

(3) Binding of complex to microtubule end:

$$M_{n-1} + TA \overset{K_i}{\rightleftharpoons} M_{n-1}TA; \quad K_i = \frac{[M_{n-1}TA]}{[M_{n-1}][TA]}$$

where the concentrations in brackets are the molar concentrations of the various species. The total concentration of tubulin, T_{total}, expressed as moles of $\alpha\beta$ tubulin dimers is given by

$$T_{total} = [T] + [TA] + [(M)] + [(M_{n-1}TA)] \tag{A2}$$

where the parentheses indicate that the concentration of assembled species is expressed in terms of moles of $\alpha\beta$ tubulin units, n, contained within the assembled species, i.e. $[(M)] = n[M]$. Now, under assembly conditions, all assembly capable dimeric tubulin (e.g. not liganded to an inhibitor) assembles into microtubules with a critical concentration Cr. Then, $[T] = Cr$, and we have

$$T_{total} - Cr = [(M)] + [(M_{n-1}TA)] + [TA] \tag{A3}$$

Turbidity is proportional to the mass of tubulin assembled into microtubules, M. At steady state, the inhibited species, $M_{n-1}TA$, are expected to be short relative to assembled microtubules. Hence, their contribution to turbidity should be negligibly small.[4] Therefore, in the presence of an inhibitor, as a close approximation, the fraction of turbidity observed relative to that in the absence of inhibitor is

$$\text{fraction of turbidity} = \frac{[(M)]}{T_{total} - Cr} = $$
$$1 - \frac{[TA] + [(M_{n-1}TA)]}{T_{total} - Cr} \tag{A4}$$

Transformation to concentrations expressed as moles of a dispersed species per liter of solution [i.e. $[(M_{n-1}TA)] = (n - 1)[M_{n-1}TA]$] and introduction of K_b and K_i from eq A1 give

$$\text{fraction} = 1 - \frac{K_b K_g^{-1}[A][1 + K_i(n - 1)[M_{n-1}]]}{T_{total} - Cr} \tag{A5}$$

Introduction of the microtubule growth equilibrium (eq A1), $[M] = [M_{n-1}]$ and $K_g = Cr^{-1} = [T]^{-1}$, gives the final inhibition equation:

$$\text{fraction} = \frac{1}{1 + K_b K_i K_g^{-1}[A]} -$$

$$\frac{K_b K_g^{-1}[A]}{(T_{\text{total}} - Cr)(1 + K_b K_i K_g^{-1}[A])} \quad (A6)$$

This equation expresses the complex interplay between the concentrations of total tubulin and free drug which are related by eq A1. Since Cr is a constant, any increase in total tubulin must increase the constituent concentrations of all the other tubulin species in the various states of assembly and liganding, as expressed by eq A3 and their inter-relation by eq A1. As a consequence, the fraction remains independent of total protein concentration at any given value of [A]. Equation A6 describes both stoichiometric and substoichiometric inhibitions. The class, in fact, is defined by the value of K_i and its combination with K_b. In the limiting case that TA does not bind to microtubules, $K_i = 0$ and eq A6 reduces to

$$\text{fraction} = 1 - \frac{K_b K_g^{-1}[A]}{T_{\text{total}} - Cr} \quad (A7)$$

Equation A7 describes inhibition by sequestration of dimeric tubulin into tubulin−drug complexes that are not capable of adding on to the end of a growing microtubule. This inhibition then must be stoichiometric. The more prevalent situation seems to be that in which the complex does bind to microtubule ends. The classification into stoichiometric and substoichiometric reflects, then, the interplay between the binding affinities of the drug to a tubulin heterodimer, K_b, and of a tubulin−drug complex to the end of a growing microtubule, K_i. Hence, one can expect a full spectrum of inhibitory capacities from strongly substoichiometric in which inhibition occurs at a very low ratio of complexed tubulin to free tubulin to fully stoichiometric at which the mole concentrations are close to equal. This is clearly seen in the pattern of inhibition by COL analogues shown in Table 1. At one extreme are TKB, COL, and ALLO, for which, at 50% reduction of turbidity, for each liganded tubulin there are 40−50 free protein molecules. At the other end are TCB and NAM, for which for each liganded tubulin there are one to two free tubulin molecules. In between are MTC, the A-C analogue of COL, for which the ratio is 17, TME (COL ring C by itself), for which 50% inhibition occurs at a ratio of one liganded tubulin per 25 free protein molecules, and TMB, for which the corresponding ratio is 5.

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