Alterations of Rings B and C of Colchicine Are Cumulative in Overall Binding to Tubulin but Modify Each Kinetic Step†

Chantal Dumortier,‡ Marina J. Gorbunoff,§ José M. Andreu,*,|| and Yves Engelborghs*,‡

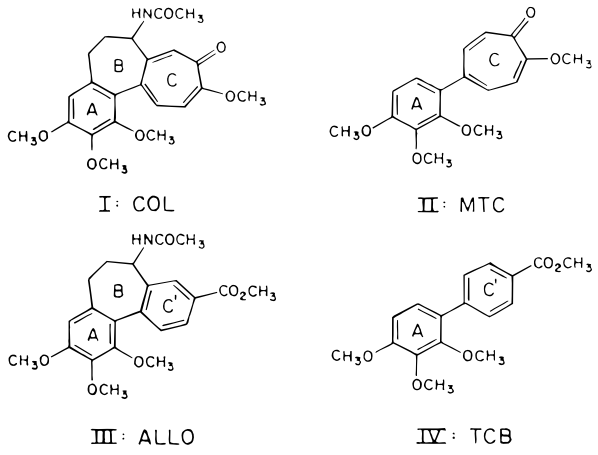
Laboratory of Chemical and Biological Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200D, B-3001 Leuven, Belgium, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, and Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, Velázquez 144, 28006 Madrid, Spain

Received June 21, 1996; Revised Manuscript Received September 25, 1996⊗

ABSTRACT: The role of the elimination of ring B and/or the modification of ring C of colchicine in tubulin binding kinetics and thermodynamics has been characterized, using four different molecules. These ligands are colchicine (COL); 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC), in which the central ring B has been reduced to one bond; allocolchicine (ALLO), in which ring C has been replaced by a six-membered ring; and 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB), where the same two modifications are made simultaneously. This paper describes the kinetics of association of ALLO with tubulin. The binding is accompanied by a fluorescence increase with slow biphasic kinetics, indicating binding to fast and slow tubulin isotypes. Binding to each of these isotypes occurs in two steps: a fast initial binding followed by a slower isomerization step. The K_1 and k_2 values for ALLO at 25 °C are 14 000 ± 2000 and 25 000 ± 6000 M^{-1} (fast and slow isotypes) and 0.055 ± 0.003 s^{-1} and 0.013 ± 0.001 s^{-1} (fast and slow isotype), respectively. For ALLO the reaction standard enthalpy change of the initial binding is 68 ± 5 kJ·mol^{-1} (fast isotype) and 45 ± 33 kJ·mol^{-1} (slow isotype) and the activation energy for the second forward step is 58 ± 14 kJ·mol^{-1} (fast isotype) and 81 ± 17 kJ·mol^{-1} (slow isotype). Displacement kinetics of bound ALLO by podophyllotoxin was monoexponential. The activation energy for the isomerization in the off direction is 107 ± 7 kJ·mol^{-1}. Comparison of the thermodynamic parameters for all four compounds shows that the modifications of both rings are cumulative with respect to overall binding. For the intermediate state there is a mutual influence of both modifications, suggesting an alteration of the reaction pathway.

In a preceding study (Dumortier *et al*., 1996), a detailed kinetic analysis of the binding of two biphenyl analogues of colchicine (TCB[1] and TKB) to tubulin showed that both molecules bind in two steps, just as colchicine does, but the binding process is faster and the affinity of the initial binding is much higher than that of colchicine. TCB and TKB are analogues closest to MTC (a bifunctional colchicine analogue lacking ring B) (Chart 1), in which the structure of ring C had been modified, while ring A was left intact. The energetic properties of the binding of TCB determined from kinetic studies are greatly different from those of colchicine (Garland, 1978; Lambeir & Engelborghs, 1981; Díaz & Andreu, 1991) and MTC (Bane *et al*., 1984; Engelborghs & Fitzgerald, 1986, 1987). These thermodynamic differences prompted the study of allocolchicine (ALLO), which is closest to colchicine in that it has three rings but in which ring C has been replaced by the same aromatic six-membered ring as in TCB (Chart 1). ALLO inhibits tubulin assembly and is a competitive inhibitor of ^{3}[H]colchicine binding (Fitzgerald, 1976; Deinum *et al.*, 1981). ALLO is weakly fluorescent in aqueous solution, and its fluorescence is dramatically enhanced by binding to tubulin, while it also quenches the intrinsic fluorescence of tubulin (Bane Hastie, 1989). It has been shown that the binding of ALLO induces in tubulin effects very similar to those that accompany colchicine binding, i.e., a weak perturbation of the protein circular dichroism, the formation of polymers of aberrant shape but with thermodynamics very similar to microtubule formation, and induction of GTPase activity (Medrano *et al.*, 1989).

Chart 1



† Research grants from I.W.T. (Belgium) to C.D. are acknowledged. We also acknowledge support of Grant DGICyT PB920007 (J.M.A.) and NIH Grant CA-16707 (M.J.G.).

* Corresponding authors.
‡ Katholieke Universiteit Leuven.
§ Brandeis University.
|| Consejo Superior de Investigaciones Cientificas.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: ALLO, allocolchicine; COL, colchicine; MES, 4-morpholineethanesulfonic acid; MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; DMSO, dimethyl sulfoxide; POD, podophyllotoxin; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl; GTP, guanosine triphosphate; GDP, guanosine diphosphate.

S0006-2960(96)01499-7 CCC: $12.00

That the association of ALLO is relatively slow has been shown by Bane Hastie (1989) and Medrano *et al*. (1989), but the full binding mechanism has not been determined. Calorimetric measurements of ALLO were done by Menendez *et al*. (1989). When compared to the overall equilibrium studies (Medrano *et al*., 1989), a detailed study of the kinetics of ALLO should reveal more details of the binding mechanism of the ligand in which ring C has been modified. Comparison of these results with those of the interactions of tubulin with colchicine, MTC, and TCB should permit a full kinetic analysis and a thermodynamic characterization of the effect of modification of ring B or/and ring C on the individual steps of binding of colchicine and their mutual influence.

MATERIALS AND METHODS

Protein. The preparation of pure tubulin and the determination of its concentration were as described previously (Dumortier *et al*., 1996).

Chemicals. ALLO was synthesized by M. J. Gorbunoff, by treatment of colchicine with sodium methoxide (Fernholz, 1950). Stock solutions were made in dimethyl sulfoxide and stored at −20 °C. For the experiments, these solutions were diluted in MES buffer adjusted to a final DMSO concentration of 5% (v/v) after 2-fold dilution in the stopped-flow apparatus. Concentrations were determined spectrophotometrically using an extinction coefficient of 11 860 M^{-1} cm^{-1} at 288 nm (Medrano *et al*., 1989). The solubility of ALLO is limited (Medrano *et al.*, 1989; Bane Hastie, 1989).

COL was purchased from Aldrich. Its concentration was determined spectrophotometrically with an extinction coefficient of 16 600 M^{-1} cm^{-1} at 350 nm.

POD was purchased from Sigma. It was dissolved in DMSO and an extinction coefficient of 3700 M^{-1} cm^{-1} at 290 nm was used (Andreu & Timasheff, 1982).

Kinetic Measurements. All kinetic studies were done with pure tubulin in a buffer consisting of 50 mM MES, 70 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM NaN_3, adjusted to pH 6.4 with NaOH and to 5% DMSO upon mixing in the stopped-flow apparatus. GDP was added to a final concentration of 1 mM to prevent polymerization. The ionic strength of this buffer is 0.1 M.

Association Kinetics. The kinetics of association were measured in a fluorescence stopped-flow apparatus (Lambeir & Engelborghs, 1981). Measurement of COL binding gave (as expected) identical results (Lambeir & Engelborghs, 1981). For the ALLO studies, excitation was done at 315 nm and emission was collected through a Kodak Wratten filter 2B (cutoff at 395 nm). The dead time of the instrument was determined with the reaction of *N*-bromosuccinimide with *N*-acetyltryptophanamide (Peterman, 1979) and was found to be around 1.5 ms. The data obtained were fitted with nonlinear least-squares curve-fitting software based on the Marquardt algorithm (Bevington, 1969).

Dissociation Kinetics. The kinetics of dissociation were followed by monitoring the loss of fluorescence of the tubulin·ALLO complex in the presence of an excess of podophyllotoxin. The dissociation of the complex was monitored on a SPEX spectrophotometer at different temperatures. The excitation monochromator (SPEX 1861 Minimate 2) was set at 315 nm. The emission monochromator (SPEX 1680 double spectrometer using two grids of 1200 lines/mm each) was set at 400 nm. All fittings were done with the program Sigmaplot, also based on the Marquardt algorithm.

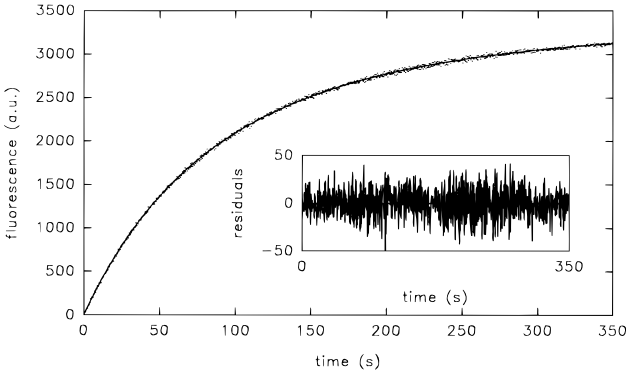


FIGURE 1: Kinetics of association of allocolchicine to tubulin. Fluorescence increase associated with the binding of allocolchicine (48 μM) to tubulin (5 μM), observed in the stopped-flow apparatus at 25 °C (excitation at 315 nm, emission above 400 nm). The experimental curve (dots) is fitted to a sum of two exponentials (line). The calculated constants are 0.020 ± 0.002 s^{-1} for the fast isotype and 0.0077 ± 0.0004 s^{-1} for the slow isotype. The inset shows the residuals between experimental and observed data.

RESULTS

(1) Stopped-Flow Association Kinetics of the Binding of ALLO to Tubulin. The binding of ALLO to tubulin results in a promotion of ALLO fluorescence, which can be used as a probe to follow the binding kinetics. The kinetics of association were studied in a stopped-flow apparatus at different temperatures under pseudo-first-order conditions in the presence of a large excess of the drug (up to 350 μM; limited by solubility). The ligand:protein ratio was at least 10:1. A typical kinetic profile for the relatively slow binding of ALLO (48 μM) to tubulin (5 μM) at 25 °C is shown in Figure 1. The kinetics were analyzed by using both one- and two-exponential fits. The fluorescence increase has to be fitted with a sum of two exponentials (biphasic kinetics), based on the values of χ^2 and on the spreading of the residuals (experimental−theoretical fit) (Figure 1):

$$F = F_\infty - \Delta F_1 \exp(-k_{obs1}t) - \Delta F_2 \exp(-k_{obs2}t) \quad (1)$$

where F_∞ is the fluorescence at infinite time and ΔF_1, k_{obs1}, ΔF_2, and k_{obs2} are the amplitudes and the observed rate constants of the fast and slow phases, respectively. The amplitude of the fast phase was about 40−50% of the total fluorescence at 25 °C.

The binding of colchicine and MTC was also best described by the above biexponential equation (Lambeir & Engelborghs, 1981; Engelborghs & Fitzgerald, 1987). The two phases are attributed to the presence of different isotypes of tubulin, which have been isolated by Banerjee and Ludueña (1987, 1991, 1992). In relation to colchicine binding the isotypes can be grouped in fast-binding isotypes ($a\beta_{II}$, $a\beta_{IV}$) and the slow-binding isotype ($a\beta_{III}$) (Banerjee et al., 1994).

The observed rate constants (k_{obs}) for the association of ALLO with tubulin were determined for different temperatures. Figure 2 shows the concentration dependence for the two phases (isotypes). Both show a very pronounced deviation from the linear relation expected for a simple

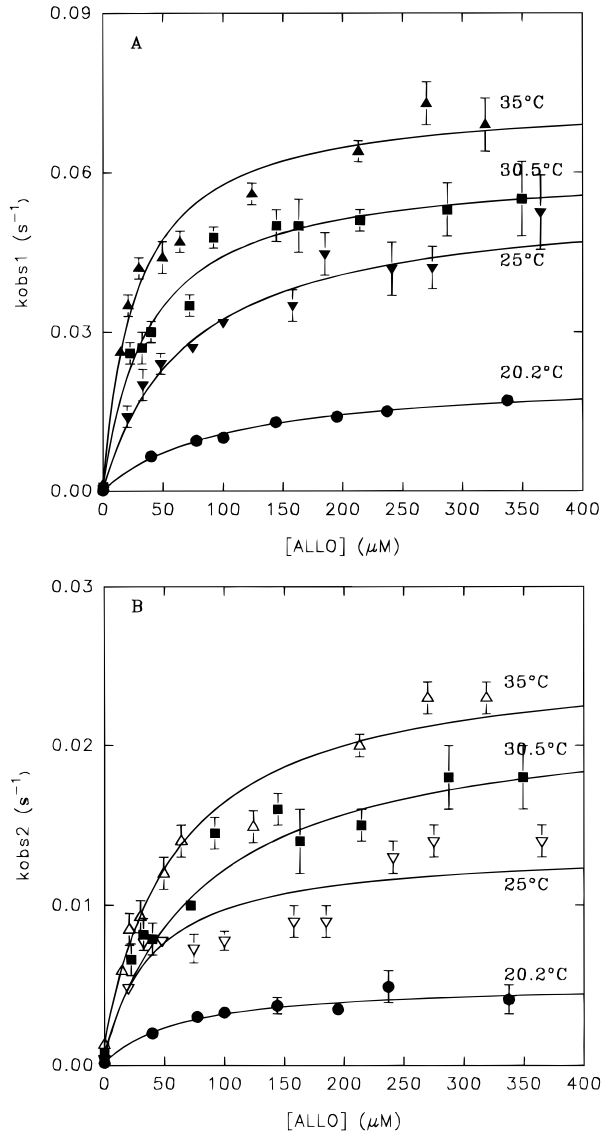


FIGURE 2: Nonlinear concentration dependence of the rate constants for the binding of ALLO to the fast (k_{obs1}, panel A) and the slow (k_{obs2}, panel B) reacting isotypes of tubulin at different temperatures (at pseudo-first-order conditions). The ligand:tubulin ratio was at least 10:1. Each point represents the mean of eight experiments. The data were fitted to the hyperbolic model. The off rate constants (k_{-2}) (intercept) were obtained separately from the displacement experiments.

pseudo-first-order concentration dependence, indicating that the binding occurs in two steps as is true for the binding of colchicine, MTC, and TCB to tubulin (Garland, 1978; Engelborghs & Fitzgerald, 1987; Dumortier *et al.*, 1996):

$$T + ALLO \overset{K_1}{\rightleftharpoons} T{\cdot}ALLO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T{\cdot}ALLO^* \qquad (2)$$

where K_1 is the equilibrium constant for the initial binding and k_2 and k_{-2} are the forward and backward rate constants for the isomerization of the initial complex (for each tubulin isotype). For such a scheme, the following rate constant can be derived for each isotype:

$$k_{obs} = k_{-2} + \frac{k_2 K_1 [ALLO]}{1 + K_1 [ALLO]} \qquad (3)$$

[In the fitting k_{-2} has the value obtained from the displacement experiments (see further)]. Measurement of the binding kinetics at different temperatures gave the thermodynamic parameters for the fast preequilibrium for the two isotypes, as well as the activation energy of the second step in the association direction (Figure 3). The thermodynamic and kinetic parameters that describe the two-step binding of ALLO to the two populations of tubulin isotypes are presented in Table 1.

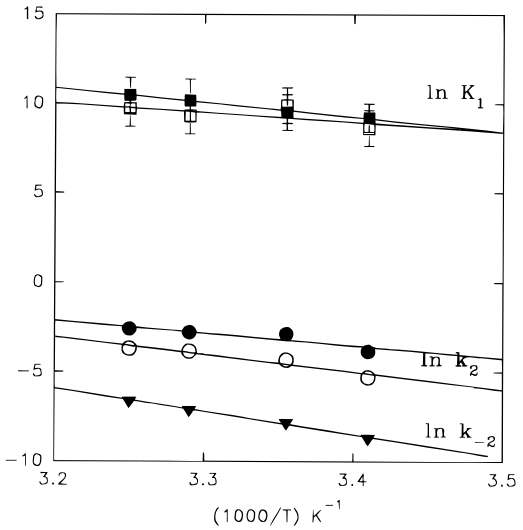


FIGURE 3: van't Hoff plots for the initial binding equilibrium (K_1) for the fast (■) and slow (□) reacting isotypes and Arrhenius plots for the association rate constants (k_2) and dissociation rate constants (k_{-2}) for the fast (filled symbols) and slow (open symbols) reacting isotypes of the ALLO complex.

(2) Dissociation Kinetics. The kinetics of dissociation were studied independently by monitoring the loss of the fluorescence of the tubulin·ALLO complex in the presence of a large excess of podophyllotoxin. Tubulin (5 μM) was incubated with ALLO (20 μM) for 20 min to form the complex. The dissociation was initiated by the addition of 1 mM POD (all final concentrations). Every dissociating molecule of ALLO is replaced by POD and the isomerization in the off direction is the rate-limiting step (k_{+POD}[POD] $\gg$ k_{+ALLO}[ALLO]). A typical fluorescence curve at 35 °C is shown in Figure 4. Surprisingly, the dissociation reaction follows single exponential (monophasic) kinetics. However, the higher noise of the curve would not allow the separation of two closely related exponentials. The backward rate constant for the isomerization (k_{-2}) at 25 °C has a value of $(3.88 \pm 0.02) \times 10^{-4}$ s^{-1}. From the temperature dependence of the displacement experiments, an activation energy of 107 ± 7 kJ·mol^{-1} for ALLO dissociation was calculated (Figure 3).

(3) Thermodynamic Parameters. With the determination of the activation energies for the forward and backward directions of the second step, all the thermodynamic and kinetic parameters for the two steps of ALLO binding become known. These are listed in Table 1. In Figure 5, the enthalpic pathway is compared with those of TCB, MTC (fast and slow tubulin isotypes), and colchicine (fast tubulin isotype).

The standard enthalpy changes for the overall bindings of the four compounds to the fast tubulin isotype are shown in Figure 6. Figure 7 shows the standard enthalpy changes for the initial and second steps of binding to the fast tubulin isotype. (Since the data of colchicine binding to the slow

Table 1: Thermodynamic and Kinetic Parameters (at 25 °C) of the Individual Steps of ALLO (Fast and Slow Isotypes), TCB, MTC (Slow and Fast Isotype), and Colchicine (Fast Isotype)

	ALLO			MTC[b]		
	fast phase	slow phase	TCB[a]	fast phase	slow phase	COL[c,d] fast phase
K_1 (M^{-1})	$(1.4 \pm 0.2) \times 10^4$	$(2.5 \pm 0.6) \times 10^4$	$(11.4 \pm 1.5) \times 10^4$	273 ± 14	1188 ± 200	220
$\Delta H°_1$ (kJ·mol^{-1})	68 ± 5	45 ± 33	44 ± 9	-1 ± 1.6	24 ± 7	-33 ± 12
$\Delta S°_1$ (J·mol^{-1} K^{-1})	303 ± 16	224 ± 116	240 ± 30	43 ± 7	142 ± 14	-63 ± 40
k_2 (s^{-1})	0.055 ± 0.003	0.013 ± 0.001	0.66 ± 0.04	58.5 ± 0.4	4.4 ± 0.4	0.3
E_{a2} (kJ·mol^{-1})	58 ± 14	81 ± 17	64 ± 2	58 ± 2	39 ± 4	100 ± 5
$\Delta H°_1 + E_{a2}$	126 ± 16	126 ± 37	108 ± 9	57 ± 2	63 ± 8	67 ± 13
k_{-2} (s^{-1})	$(3.88 \pm 0.02) \times 10^{-4}$		0.25 ± 0.05	0.058 ± 0.01	0.025 ± 0.01	5.3×10^{-6}
E_{a-2} (kJ·mol^{-1})	107 ± 7		86 ± 20	81 ± 6	74 ± 6	94 ± 10
$\Delta H°_2$ (kJ·mol^{-1})	-49 ± 21	-26 ± 18	-22 ± 20	-23 ± 6	-35 ± 7	6 ± 11
$\Delta S°_2$ (J·mol^{-1} K^{-1})	-124 ± 21	-58 ± 40	-66 ± 20	-20 ± 6	-74 ± 8	111
$\Delta H°_{tot}$(kJ·mol^{-1})	19 ± 16	19 ± 37	22 ± 21	-24 ± 7	-11 ± 10	-27 ± 16

[a] From Dumortier et al (1996). [b] From Engelborghs and Fitzgerald (1987). [c] From Lambeir and Engelborghs (1981) and Díaz and Andreu (1991). [d] The data for colchicine binding to the slow isotype are not sufficiently accurate.

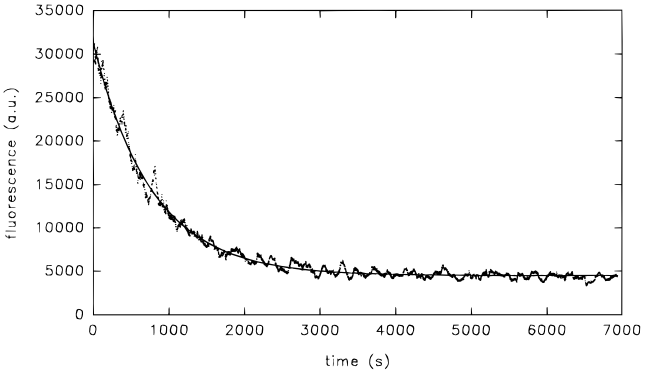


FIGURE 4: Displacement of bound ALLO by excess podophyllotoxin at 35 °C. Tubulin (5 μM) was incubated with ALLO (20 μM) for 20 min to form the complex. The dissociation was started by the addition of 1 mM podophyllotoxin. The experimental curve is fitted to a one-exponential function.

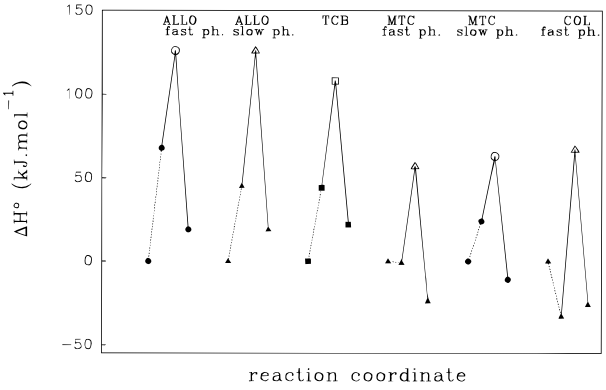


FIGURE 5: Reaction path for the binding of tubulin to allocolchicine, TCB, MTC, and colchicine. The open symbols represent the transition state for the second step. For colchicine only data for the fast isotype are available; for MTC and ALLO data are available for both isotypes. TCB does not sense the difference.

isotype are lacking, no complete comparable graph can be made for the slow isotype.)

DISCUSSION

Detailed kinetic stopped-flow studies have been described for the interaction of tubulin with colchicine (Garland, 1978; Lambeir & Engelborghs, 1981), with MTC, a bifunctional analogue of colchicine that lacks the middle ring (modification −B) (Engelborghs & Fitzgerald, 1986, 1987) and with the biphenyl analogue TCB, in which the tropolone methyl

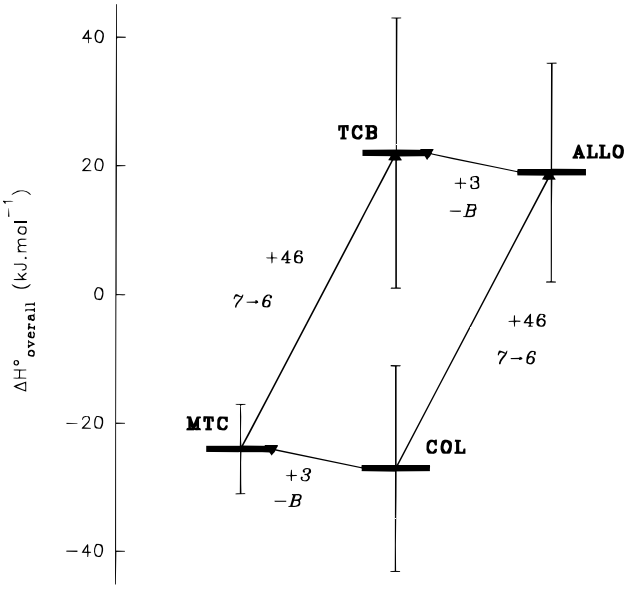


FIGURE 6: Standard enthalpy changes ($\Delta H°_{overall}$) of global binding of the four compounds to the fast tubulin isotype.

ether ring (ring C) had been transformed into a (*p*-carbomethoxy)benzene (modification 7 → 6 and modification −B simultaneously) (Dumortier *et al*., 1996). The finding that TCB exhibits a much higher affinity in the first step of binding (relative to colchicine) suggests that the first step contains a contribution from the whole molecule (instead of ring A or ring C alone). In this paper, the kinetics of the colchicine analogue in which ring C has been replaced by a six-membered ring, allocolchicine, are described. This permits the characterization of the 7 → 6 modification and a full kinetic and thermodynamic comparison of the four molecules.

(1) Kinetic Analysis of the Binding of ALLO to Tubulin. It has been shown by Bane Hastie (1989) and Medrano *et al*. (1989) that binding to tubulin promotes enhancement of ALLO fluorescence and that the association with tubulin is relatively slow, but the full mechanism of binding was not determined. Here, a detailed kinetic study at different temperatures has been done. The kinetic behavior of ALLO is formally very similar to that of colchicine and MTC. Under pseudo-first-order conditions, two phases are observed (biphasic kinetics). For colchicine, it has been shown that the two parallel phases are caused by separable isotypes (Banerjee & Ludueña, 1987, 1991, 1992). In the case of TCB,

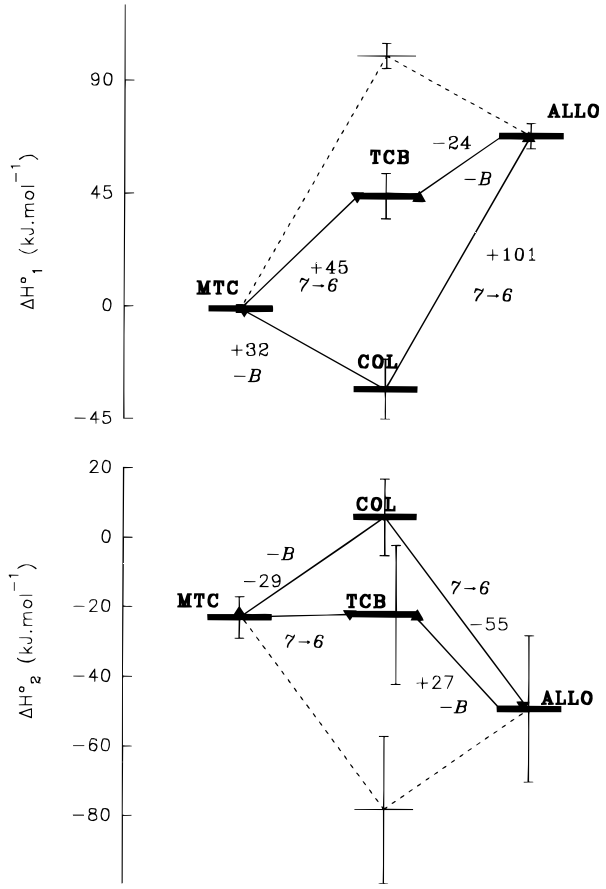


FIGURE 7: Standard enthalpy changes of the initial ($\Delta H°_1$, top panel) and second ($\Delta H°_2$, bottom panel) step of binding to the fast tubulin isotype. The dotted lines represent the $\Delta H°$ values for TCB if pure additivity were applicable.

which is identical to ALLO except for the absence of the ring B, the binding is monophasic, which indicates that this drug does not discriminate between the isotypes of tubulin (Dumortier *et al.*, 1996).

In the case of ALLO binding, the nonlinearity of the concentration dependence of the two observed rate constants can again be explained by a two-step binding model, which involves a fast preequilibration step, followed by a slow isomerization to form a stable fluorescent complex.

The kinetic measurements show that the affinity of the initial step for ALLO is high ($14\,000 \pm 2000$ M^{-1} for the fast reacting isotype and $25\,000 \pm 6000$ M^{-1} for the slow-reacting isotype at 25 °C), but the affinity of the second step is much lower (142 ± 7 for the fast isotype). This is analogous to TCB binding [$K_1 = (11.4 \pm 1.5) \times 10^4$ M^{-1}; $K_2 = 2.6 \pm 0.5$) (Dumortier *et al.*, 1996) but is highly different from colchicine binding (K_1 is on the order of 200 M^{-1} and K_2 is on the order of 55 000) (Lambeir & Engelborghs, 1981) and MTC binding (Engelborghs & Fitzgerald, 1987). The high affinity of the initial binding and the low value of K_2 for ALLO and TCB strongly suggest that both rings A and C contribute to the binding in the first step.

The overall binding constant of ALLO [$(19 \pm 3) \times 10^5$ M^{-1} for the fast-reacting isotype] calculated from the on and off rate constants ($K_b = K_1K_2$) agrees fairly well with the value (12 ± 5) $\times 10^5$ M^{-1} measured by equilibrium ligand fluorescence (Medrano *et al.*, 1989).

Table 2: Kinetic Analysis (at 25 °C) of the C into C′ Modification and Elimination of Ring B[a]

modification	γK_1	γk_2	γk_{-2}	γK_2
7 → 6				
(COL → ALLO)	×64	÷5.4	×73.2	÷399
(MTC → TCB)	×414	÷88.6	×4.3	÷380
−B				
(COL → MTC)	×1.24	×195	×10943	÷56
(ALLO → TCB)	×8.1	×12	×644	÷54

[a] γX(ligand 1 → ligand2) is the factor of X going from ligand 1 to ligand 2. Only fast-phase data are compared.

The initial binding of ALLO is endothermic, as is the case with TCB. The initial binding of ALLO (and TCB) is entropy-driven. It should be noted that the activation energy reported by Bane Hastie (1989) (80 ± 6 kJ·mol^{-1}) was determined from the apparent bimolecular rate constant for the fast phase at a single ALLO concentration and is the sum of the activation energy of the second step and the enthalpy change for the fast preequilibrium. The present slightly higher value of the activation energy ($\Delta H°_1 + E_{a2}$) measured by kinetic experiments (126 ± 16 kJ·mol^{-1}) (see Table 1) is somewhat higher than the values reported for colchicine by Lambeir and Engelborghs (1981) and for MTC by Engelborghs and Fitzgerald (1987).

(2) Comparison of the Kinetic Parameters for the Binding of ALLO, TCB, MTC, and COL to Tubulin. Modifications in the colchicine molecule have marked effects on the binding kinetics. Elimination of ring B (COL → MTC and ALLO → TCB) accelerates the binding process, due to a faster second step (lowering of the activation energy, Ea_2, for the COL_MTC transformation) and the fact that the initial complex is not trapped in a deep negative enthalpy trough (see Tables 1 and 2). An explanation that had been offered is that the colchicine and allocolchicine bindings require a conformational change in the ligand to one that can fit sterically into the tubulin binding site (Detrich *et al.*, 1981; Brossi *et al.*, 1990). MTC, which lacks ring B, can assume an active conformation very quickly due to the free rotation about the phenyl−tropone bond and binds rapidly to tubulin (Bane *et al.*, 1984). The same explanation can be used for the fast binding process of TCB. This explanation can be questioned, however, on the basis of the demonstration that colchicine retains its solution conformation in its complex with tubulin (Bane Hastie, 1991). Other hypotheses are that ring B inhibits sterically the tubulin conformational change in the second step of the binding process (Ray *et al.*, 1981; Bhattacharyya *et al.*, 1986), or that ring B constitutes a steric impediment to the approach of ring A and/or ring C to their binding sites and to their dissociation from the protein (Andreu *et al.*, 1991). Recently, kinetic studies of several analogues of the middle ring of colchicine have shown that this ring is responsible for the overall energetic barrier, which is a function of the electronic nature of the substituent in position C_7 (Pyles & Bane Hastie, 1993).

The transformation of the tropolone methyl ether ring into a carbomethoxybenzene ring (7 → 6 modification) (COL → ALLO and MTC → TCB) induces a strong enhancement of K_1, a limited slowing down of k_2 (a positive ΔE_{a2} for MTC → TCB, but negative ΔE_{a2} for COL → ALLO; see Table 1) and an acceleration of the dissociation process (see Table 2). In both systems the affinity of the second step is decreased 400-fold by the transformation (see Table 2).

(3) Comparison of the Thermodynamic Parameters for the Binding of ALLO, TCB, MTC, and COL to Tubulin. When the enthalpy patterns (Figure 5) of the different compounds are compared, one feature is immediately apparent: the patterns belong to two families, the ALLO/TCB family and the COL/MTC family. This points to the fact that the major effect is the ring C modification, which increases the enthalpy level of the initial complex, the final complex, and the transition state in between the two; i.e., it makes the initial and global bindings endothermic. The consequence of the −B modification is different for the two families: for the COL → MTC modification, its major feature is the rise of the enthalpy level of the intermediate and a small decrease of that of the transition state. For the ALLO → TCB modification, it reduces the enthalpy levels of the intermediate (fast isotype) and the transition state.

A more detailed comparison of $\Delta H°_{overall}$ values is given in Figure 6. It shows that the overall enthalpy changes are cumulative, as also shown for the free energy changes from the equilibrium measurements by Medrano *et al*. (1989). Figure 6 also shows that the contribution of ring B (in going from MTC to COL or from TCB to ALLO) to the overall enthalpy change is very small, again in agreement with previous statements (Andreu *et al*., 1991). In other words, we can say that for the overall binding process the effect of the modification of ring C is the same whether ring B is present or not; i.e., the consequences of modification of either ring B or C are mutually independent. The kinetically estimated overall enthalpy change of ALLO (19 ± 16 kJ·mol^{-1}) is significantly different from the direct calorimetric measurements (−10.8 ± 2 kJ·mol^{-1}) (Menendez *et al*., 1989) and from the van't Hoff analysis (Medrano *et al*., 1989). This, however, does not modify the conclusion that the consequences of the modifications are independent and not linked to each other in the overall binding.

It is now interesting to make the same comparison for the individual binding steps. This justifies the kinetic studies that are the only way to get information about the individual steps. In fact, there can be two possible outcomes and it is not possible to predict which one is going to be true: (a) the rings behave independently in the individual steps or (b) there is an interdependence. Construction of plots similar to that of Figure 6 for $\Delta G°_1$ and $\Delta G°_2$ indicates that the effects of the modifications are mutually independent within experimental error in free energy terms. Furthermore, it shows that the only significant free energy contribution of ring B takes place in the second step and amounts to −10 kJ·mol^{-1}. This was suggested by Andreu *et al*. (1984) to arise from the relative immobilization of the intramolecular rotation of the biaryl bond of MTC upon the binding of rings A and C with the loss of rotational entropy. In COL this bond is fixed in proper steric orientation by ring B. Similar plots have been constructed for $\Delta H°_1$ and $\Delta H°_2$ for the fast tubulin isotype. These are shown in Figure 7. A mutual interdependence of the contributions of the two modifications (alteration of ring C or excision of ring B) is seen for both steps of the binding process, as the effects of the individual modifications have different magnitudes that depend on the order of the structural transitions (7 → 6 then −B, or −B then 7 → 6). Since addition of $\Delta H°_1$ and $\Delta H°_2$ at each transformation gives the symmetrical box of Figure 6, this interdependence does not signify cooperativity or linkage in a thermodynamic sense. The simplest interpretation is that, in the binding of these ligands, the extent of contact formed between the drug and tubulin in each step differs from one molecule to another. For example, alteration of ring C (7 → 6) involves in the first step different changes from one molecule to another in the extent of intermolecular contact, whether the modification is made in the three-ring system (COL → ALLO) or in the two ring system (MTC → TCB). The second step, then, encompasses the balance of the intermolecular contacts involved in the complexing, which by necessity will be different whether the modification is ALLO → TCB or COL → MTC, since the overall effect on the binding enthalpy change must be independent of the order of the structural modifications, i.e., COL → ALLO → TCB or COL → MTC → TCB.[2]

For comparison, the dashed lines in Figure 7 show the expected values of $\Delta H°_1$ and $\Delta H°_2$ for TCB if the enthalpic contribution of the two modifications encompassed in COL → TCB were equal to COL → ALLO plus COL → MTC, which would assume that all the ligands make identical extents of molecular contact with tubulin in each of the two steps. Once again, it is clear that this is not the case.

Figure 7 also shows that the enthalpic contribution of all modifications is destabilizing for the initial complex and stabilizing for the final complex when compared with colchicine. However, the free energy changes lead to stabilization of the intial complex (except for MTC) and destabilization of the final complex. The higher affinities of the modified molecules in the first step clearly come from a positive entropy contribution in the first step, again compensated by a negative differential entropy change in the second step (see Table 1).

On the other hand, the overall thermodynamic pattern of TKB binding to tubulin (fast and slow isotypes give the same) is very similar to that of colchicine (Dumortier *et al*., 1996). This seems to be in contrast with the comparison presented above. The only conclusion that can be made from this comparison is that the 7 → 6 modification in TKB is different from the one in TCB and that this difference in C-ring structure compensates a number of effects observed in TCB. In fact, a structural study has shown that TKB can fit into the COL binding site in a manner similar to the parent molecule, while the $COOCH_3$ group of TCB sterically prevents TCB from doing so (Rossi *et al*., 1996). Thus, a span of about 80 kJ·mol^{-1} in enthalpy change and about 300 J·K^{-1}·mol^{-1} in entropy change can be the consequence of the removal of one oxygen atom.

(4) Conclusions. In this paper, we have presented the binding mechanism and kinetics of association and dissociation of ALLO to tubulin: it binds in two steps (as do COL, MTC, and TCB), the association and dissociation processes are faster than COL, and the affinities of the first and second steps are changed. From the comparison of the kinetic and thermodynamic parameters of ALLO with those of TCB, MTC, and COL, the following points become clear: (a) The ring C to ring C′ transformation results in an acceleration of the dissociation process and in a significant increase of the

[2] An alternative proposal would be that some tubulin−ligand contacts (such as a hypothetical ring B contact) are made in the first step which are dissociated in the second, and that these are different for the different ligands. However, at present there is no evidence for this complication (but on the contrary, ring B is considered to make no contribution in the ground state; Pyles & Hastie, 1993).

enthalpy levels of all states, relative to the reference. (b) The absence of ring B relieves a kinetic barrier to binding and dissociation in the second step by lowering the enthalpy level of the transition state. Concerning the intermediate state, the absence of ring B raises the enthalpy level when going from COL to MTC and decreases the enthalpy level when going from ALLO to TCB. (c) The effects of the modification of ring B on ring C (and vice versa) are independent for the overall enthalpy change but interdependent for the individual steps, which suggests that they modify the mechanistic reaction pathway.

These results clearly show that a kinetic study can reveal fascinating aspects of this remarkable binding process.

ACKNOWLEDGMENT

We thank Dr. S. N. Timasheff for clarifying discussions on the manuscript.

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BI9614996