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Thermodynamics of Colchicinoid-Tubulin Interactions

ROLE OF B-RING AND C-7 SUBSTITUENT*

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The quenching of tryptophan fluorescence has been used to determine the kinetic and thermodynamic parameters of binding of B-ring analogs of colchicine to tubulin. The on rate, activation energy, off-rate, and thermodynamics of binding reaction have been found to be controlled at different points of analog structure. The on-rate and off-rate of deacetamidocolchicine (DAAC) binding with tubulin is 17 times slower than that of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone-tubulin (AC-tubulin) interaction, although both reactions have very similar activation energies. The presence of B-ring alone does not significantly affect the thermodynamics of the binding reactions either, since both AC-tubulin and DAAC-tubulin interactions are enthalpy driven. Introduction of a NH_2 group at C-7 position of the B-ring, as in deacetylcolchicine (NH_2-DAAC) lowers the on-rate further with a significant rise in the value of the activation energy. However, bulkier substitutions at the same position, as in demecolcine (NHMe-DAAC) and *N*-methyldemecolcine (NMe_2-DAAC) have no significant additional effect either on the on-rate or on the value of activation energy. Introduction of NH_2 group in the C-7 position of B-ring also increases the positive entropy of the binding reaction to a significant extent, and it is maximum when NMe_2 is substituted instead of NH_2 group. Thus, interaction of NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC with tubulin are entropy driven. Our results suggest that the B-ring side chain of aminocolchicinoids makes contact(s) with dimeric tubulin molecules.

Colchicine, the major alkaloid in *Colchicum autumnale*, is medically used for the treatment of gout (1) and Familial Mediterranean fever (2). Due to its immense therapeutic importance, a large number of colchicine and thiocolchicine analogs have been synthesized and tested for their biological activities (3–5). Colchicine exerts its antimitotic property upon binding to a high affinity site on the tubulin heterodimer (6–8). It is composed of a trimethoxybenzene ring (A-ring), a methoxytropone ring (C-ring), and a seven-membered ring (B-ring), which anchors the A- and C-rings (Fig. 1). Structure activity studies indicate that the A- and C-rings of colchicine comprise the minimum structural features of the molecule necessary for its high affinity binding to tubulin. Insertion of a bulky group in the A-ring of colchicine, as in colchicoside, causes complete loss of binding, indicating that the requirement of the A-ring is stringent (4). On the other hand, several changes in the C-ring such as different substitutions at the C-10 position or a replacement of the seven-membered ring with a six-membered ring are tolerated (9–14). Colchicine analogs modified at, or depleted of, the B-ring are known to retain potent antimitotic activity, self-assembly inhibitory activity, and the binding activity to tubulin at the colchicine site (15–17). Nevertheless, the presence of B-ring alone, or substituents at C-7 position, influences the on-rate, activation energy, off-rate, reversibility, and the quantum yield of the complexes of the drug with tubulin (9, 16–22). The thermodynamic contributions of A- and C-rings of colchicine in its binding to tubulin have also been reported. Binding of tropolone methyl ether (a C-ring analog) is characterized by negative apparent enthalpy and entropy changes, whereas *N*-acetylmescaline (an A-ring analog) interaction with tubulin has positive enthalpy and entropy changes (23). Binding of AC, a simple bifunctional ligand containing A- and C-rings with tubulin has been found to be enthalpy driven (17). Studies on the binding thermodynamics of colchicine-tubulin interaction have provided conflicting results. While early equilibrium studies on colchicine-tubulin interaction reported high entropy value for the binding reaction, calorimetric and kinetic studies reported the negative enthalpy value for the same interaction (8, 20, 24, 25).

In the present study, we have determined the thermodynamic parameters for the binding reactions of four B-ring analogs of colchicine with tubulin: deacetamidocolchicine (DAAC),[1] deacetylcolchicine (NH_2-DAAC), demecolcine (NHMe-DAAC), and *N*-methyldemecolcine (NMe_2-DAAC). Our study indicates that the presence of B-ring *per se* does not affect the entropic contribution significantly, as binding of both AC and DAAC are enthalpy-driven reactions. It is the amino substituent at the C-7 position in the B-ring that converts an enthalpy-driven reaction into an entropy-driven reaction. Our thermodynamic data of colchicinoid-tubulin interactions suggest that the C-7 substituent on the B-ring of the colchicinoids studied here make additional contact(s) with the dimeric tubulin molecule.

EXPERIMENTAL PROCEDURES

Pipes, GTP, EGTA, colchicine, and demecolcine were purchased from Sigma. Deacetylcolchicine and colchicine fluorescein were obtained from Molecular Probes, Inc. All other reagents used were of analytical grade. Other colchicine analogs were gifts from T. J. Fitzgerald (Florida A & M University) and Susan Bane Hastie (SUNY, Binghamton).

Goat brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly-disassembly in PEM buffer (0.05 M Pipes, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.9, at 25 °C) in presence of 1 mM GTP followed by two more cycles in 1 M glutamate buffer (26) and stored at −70 °C. The concentration of protein was determined by the method

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[1] The abbreviations used are: DAAC, deacetamidocolchicine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); AC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone; NH_2-DAAC, deacetylcolchicine; NHMe-DAAC, demecolcine; NMe_2-DAAC, *N*-methyldemecolcine.

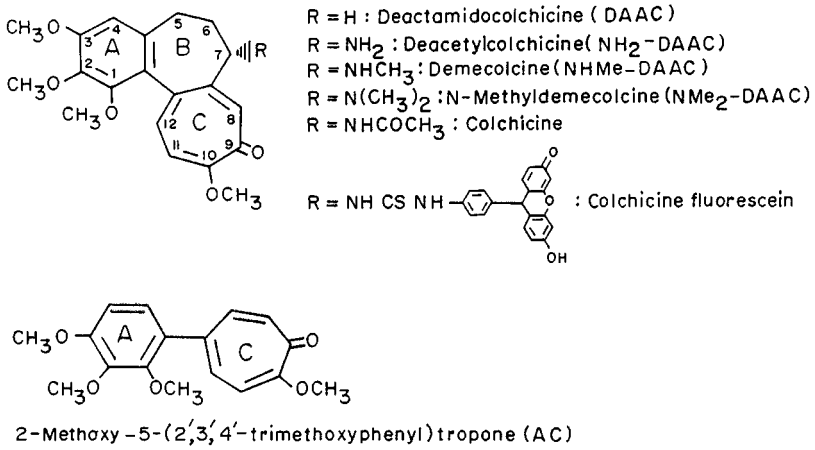


FIG. 1. **Structure of colchicine, AC, and B-ring analogs of colchicine.**

of Lowry *et al.* (27).

Stock solutions of colchicine and its analogs were prepared either in water or in dimethyl sulfoxide (Me_2SO). The maximum concentration of Me_2SO in the reaction mixture was 5% for DAAC and was less than 1% for other colchicinoids. The concentrations of the ligands were determined from the respective extinction coefficients (22).

Association Kinetics—The kinetics of the association of colchicinoids with tubulin were measured under pseudo-first-order conditions using a Hitachi F-3000 spectrofluorometer. Concentrations of tubulin and colchicinoids were 1 and 20 μM, respectively. The ligand was added to tubulin solution, and emission at 336 nm was measured upon excitation at 295 nm (excitation slits = 5 nm). Under these experimental conditions, no aggregation of tubulin was detected by the size exclusion high pressure liquid chromatography column (22). The temperature was controlled with a circulating water bath accurate to ±0.5 °C. The quenching data were analyzed according to Pyles and Bane Hastie (22) using the following biexponential equation:

$$F = Ae^{-k_1 t} + Be^{-k_2 t} + C \qquad \text{(Eq. 1)}$$

where F is the fluorescence of the ligand-tubulin complex at time t, A and B are the amplitudes for the fast and slow phases, k_1 and k_2 are the pseudo-first-order rate constants for these two phases, respectively, and C is an integration constant. As, for all the tubulin-colchicinoid complexes, the amplitude of the slow phase, B, was small relative to that of the fast phase, A, the slow phase was not analyzed further (28). The apparent second-order rate constant (k_{on}), was obtained by dividing the observed rate constant for the fast phase (k_1) by the ligand concentration. Association rate constants were determined at different temperatures ranging from 17 to 37 °C.

Dissociation Kinetics—The dissociation of colchicinoid-tubulin complexes were measured by monitoring the time-dependent increase of intrinsic protein fluorescence as the ligand was released from its binding site on tubulin upon a 300-fold dilution of the complex (17). This process was described as a single first-order reaction. The rate constant of this process was determined using the relation

$$Ln\,(F_\infty - F) = k_{off}\,t + \text{const} \qquad \text{(Eq. 2)}$$

where F_∞ and F are the maximum intrinsic protein fluorescence intensity at infinite time and at time t, respectively, and k_{off} is the first-order dissociation rate constant. Dissociation rate constants were determined at different temperatures ranging from 17 to 37 °C.

Scatchard Analysis—Scatchard analysis of DAAC-tubulin interaction was performed at different temperatures ranging from 17 to 37 °C according to Banerjee *et al.* (29).

Data Analysis—Equation 3 can be written as follows:

$$F = \frac{r(F_o - F_\infty)}{r+1}e^{-k_1 t} + \frac{F_o - F_\infty}{r+1}e^{-k_2 t} + F_\infty \qquad \text{(Eq. 3)}$$

where F_o is the fluorescence at time zero, F_∞ is the fluorescence when saturation was reached, and $r = A/B$. The unknown parameters are k_1, k_2, F_o, F_∞, and r. Each of these was systematically varied within a given range, and statistical estimates of the quality of fit for obtained F with the experimentally determined curve were performed for each point of iteration. The parameters obtained for the best-fit curve giving minimum χ^2 value were thus calculated using a BASIC program written for this purpose. For dissociation kinetics, the two unknown parameters k_{off} and F_∞ were varied and the best-fit values giving minimum χ^2 were obtained by similar iteration on Equation 2.

RESULTS AND DISCUSSION

Association and Dissociation Kinetics—In the present study, the association rate constants of DAAC, NH_2-DAAC, NHMe-DAAC, NMe_2-DAAC, and colchicine (see Fig. 1 for structures) with tubulin were determined by drug-induced quenching of tubulin fluorescence. Fig. 2 *inset* shows a representative kinetic profile for the binding of NHMe-DAAC to tubulin at 25 °C. Apparent second-order rate constants (k_{on}) at different temperatures were determined from bi-exponential curves for fluorescence *versus* time, fitted to experimental data as described "Experimental Procedures." k_{on} for the various ligands interacting with tubulin were plotted against $1/T$ and are shown in Fig. 2. The second-order rate constants for the fast phase at 37 °C are presented in Table I, along with the calculated values of activation energies ($E_{a'}$) and the pre-exponential factor (A) obtained from Fig. 2. Values of second-order rate constants and activation energies of deacetamidocolchicine and three aminocolchicinoids are in good agreement with those determined by Pyles and Hastie (22) for the calculation of thermodynamic parameters in the transition states. A comparison of the association rates of AC and DAAC clearly reveals that the B-ring itself has a dramatic effect on the association rate, although the activation energies of binding of both drugs are identical. According to Arrhenius equation, the rate constant is a product of the activation energy term (E_a) and the pre-exponential factor A, *i.e.* $k_{on} = A{\cdot}e^{-E_a/RT}$. While the activation energy measures the temperature sensitivity of a reaction (determined from the slope of the activation energy curve), A is related to activation entropy. Thus, two reactions with different rate constants might have the same A value but different activation energies and *vice versa*. We calculated the values of A for AC and DAAC binding to tubulin from the Arrhenius plot and found that it is about 70 times higher for AC compared to DAAC. The activation entropy is related to the pre-exponential factor A by the following equation (30):

$$A = e^{-\Delta n} \cdot kT/h \cdot e^{(\Delta S)^{\ddagger}/R} \qquad \text{(Eq. 4)}$$

where Δn is the change in the number of molecules when the complex is formed and $(\Delta S)^{\ddagger}$ is the activation entropy. Transition state free energy, enthalpy, and entropy of AC and DAAC binding to tubulin have recently been measured (22). The free energy and enthalpy values in the transition states are very close for both AC and DAAC, whereas entropy values differ significantly (22). This difference in the activation entropies of AC-tubulin and DAAC-tubulin interaction probably arises from the restriction imposed upon DAAC by the presence of the

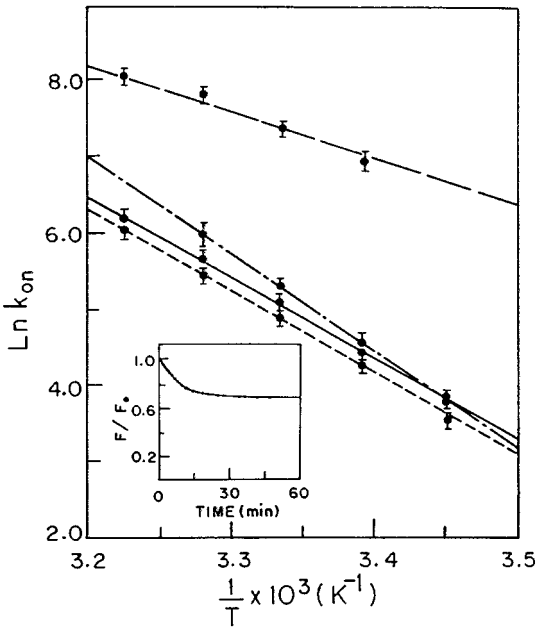


FIG. 2. **Effect of temperature on the apparent second-order rate constant of colchicine B-ring analogs binding to tubulin.** Binding studies were carried out with DAAC (—- —- —-), NH_2-DAAC (— - — - —), NHMe-DAAC (– – –), NMe_2-DAAC (——). *Inset*, quenching of intrinsic protein fluorescence upon NHMe-DAAC binding to tubulin. NHMe-DAAC (final concentration, 20 μM) was added to tubulin (concentration, 1 μM). Kinetics was followed for 1 h at 25 °C by measuring the intensity of intrinsic protein fluorescence. Excitation and emission wavelengths were 295 and 336 nm, respectively.

TABLE I
Association and dissociation rate constants and activation energies of binding of colchicine and its B-ring analogs to tubulin

Ligand	k_{on}[a]	$E_{a'}$[b]	$A \times 10^{-2}$[c]	$k_{off} \times 10^3$[a]
	$M^{-1}s^{-1}$	*kcal mol*$^{-1}$	$M^{-1}s^{-1}$	s^{-1}
Colchicine	138 ± 2[d]	19.3 ± 0.1[d]	4.03[e]	0.018[f] (0.0053)[g]
NMe_2-DAAC	485 ± 39	21.0 ± 1.8	6.32	2.6 ± 0.2
NHMe-DAAC	458 ± 72	22.6 ± 0.6	5.58	2.0 ± 0.2
NH_2-DAAC	560 ± 41	25.4 ± 2.0	10.96	3.3 ± 0.5
DAAC	3031 ± 207	12.3 ± 1.3	35.51	3.0 ± 0.6
AC[h]	52000.0	13	2430	60.0

[a] Determined at 37 °C.
[b] $E_{a'}$, the activation energies.
[c] *A*, the pre-exponential factor of Arrhenius equation.
[d] From Pyles and Bane Hastie (22).
[e] Unpublished observation from this laboratory.
[f] From Diaz and Andreu (25).
[g] From Garland (42).
[h] Obtained from data previously published by Bane *et al.* (17).

B-ring and thus accounts for the difference in pre-exponential factors (Table I).

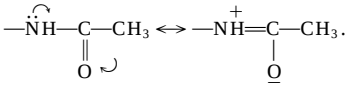
The effect of substitutions on the B-ring on the rate constants, activation energies, and *A* values of the binding of DAAC and aminocolchicinoids are compared in Table I. A significant drop in the association rates and a significant enhancement in the activation energies is apparent when NH_2 group is present at the C-7 position of the B-ring. However, substitution by further bulky group(s) (*e.g.* NHMe and NMe_2 groups) did not affect either the association rate or the activation energy. What really happens to the binding process when $-NH_2$ is substituted at the C-7 position in the B-ring is difficult to understand from the present state of knowledge of B-ring analogs binding to tubulin. It is to be noted that whereas colchicine is highly soluble in water, other aminocolchicinoids studied here are weakly soluble, indicating that the solvation property of those

TABLE II
Equilibrium constants and thermodynamic parameters of tubulin-colchicinoid complexes

Ligand	$K_a \times 10^{-4}$	ΔG	ΔH	ΔS^o
	M^{-1}	*kcal mol*$^{-1}$	*kcal mol*$^{-1}$	*cal K*$^{-1}$ *mol*$^{-1}$
Colchicine[a]	300[a]	−9 to −10 (−10 ± 0.02)[b]	+10 to +16 (−6.3 ± 3.1)[b]	60 to 80 (16.3 ± 1.7)[b]
NMe_2-DAAC	18.5 ± 0.08	−7.5 ± 0.2	+9.52 ± 0.50	54.9 ± 0.9
NHMe-DAAC	16.9 ± 0.13	−7.7 ± 0.3	+6.66 ± 2.4	46.3 ± 7.8
NH_2-DAAC	21.2 ± 0.11	−7.6 ± 0.3	+6.66 ± 1.0	46.5 ± 3.4
DAAC	104.0 ± 1.6	−8.6 ± 0.5	−3.68 ± 0.50	15.8 ± 1.6
AC[c]	35.0	−7.90	−6.80	3.60

[a] Values were obtained from Bhattacharyya and Wolff (8) and Bryan (24).
[b] Thermodyanamic data from Diaz and Andreu (25).
[c] From Bane *et al.* (17).

molecules are very much influenced by the side chain at C-7 position. The presence of lone pair of electrons of nitrogen will significantly influence the carbonyl oxygen of colchicine side chain as follows:



SCHEME I.

Thus, the oxygen of the colchicine side chain and the electron rich nitrogen atom in aminocolchicinoids can serve as potential electron donor in making hydrogen bond with surroundings. It is possible that the amino and carbonyl groups are involved in making important contacts with the protein in the complex form. Recently, it was proposed that the substituents in the B-ring of aminocolchicinoids point away from the colchicine binding site toward the exterior of the protein during their interaction with tubulin (22). Our thermodynamic data also support this proposition (see below). We observe a dramatic change in the enthalpy and the entropy of the tubulin binding reaction of DAAC when compared with that of other aminocolchicinoids (Table II).

Dissociation rate constants were determined by measuring the enhancement of intrinsic protein fluorescence due to release of colchicinoids from tubulin-colchicinoid complexes upon a 300-fold dilution of the complex by PEM buffer. This method has been successfully used previously to determine the dissociation kinetics of AC-tubulin complexes (17). The dissociation rate constants were determined using Equation 2 (see "Experimental Procedures"). The temperature dependence of dissociation rate constants for DAAC, NH_2-DAAC, NHMe-DAAC, and NMe_2-DAAC were also determined by the same procedure. A comparison of dissociation rate constants (see Table I) indicates that the presence of B-ring significantly lowers the dissociation rate of drug-tubulin complexes. The dissociation rates for DAAC-tubulin complex and aminocolchicinoid-tubulin complexes are 18–30-fold less than that of AC-tubulin complex (Table I). However, the first-order dissociation rates for DAAC-tubulin and aminocolchicinoid-tubulin complexes are comparable and are about 100–150-fold higher than that of the colchicine-tubulin complex. It was suggested previously that the >C=O in the side chain is responsible for the poor reversibility of the colchicine-tubulin interaction (19). Dissociation rate of another colchicine analog wherein >C=O was substituted by >C=S (colchicine fluorescein) (see Fig. 1) was determined under identical conditions. The rate constant (1.6×10^{-3} s^{-1} at 27 °C) was similar to those obtained for DAAC or other aminocolchicinoids and much greater than that for colchicine, supporting the earlier hypothesis (19).

Equilibrium Constant and Thermodynamic Parameters—

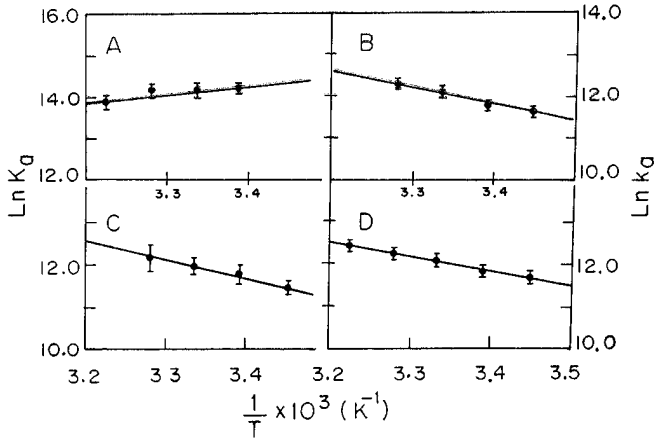


FIG. 3. **Effect of temperature on the equilibrium constants of B-ring analog-tubulin interactions.** van't Hoff plots of the reaction of tubulin with DAAC (*A*), NH_2-DAAC (*B*), NMe_2-DAAC (*C*), and NHMe-DAAC (*D*) are shown.

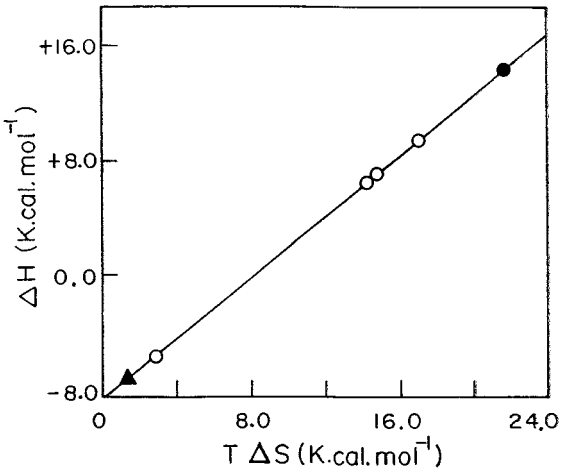


FIG. 4. **Plot of ΔH *versus* $T\Delta S$ for binding of tubulin with colchicine analogs at 37 °C.** Data for colchicine-tubulin (●) and AC-tubulin (▲) complexes were obtained from Bryan (24) and Bane *et al.* (17), respectively. Data for aminocolchicinoid-tubulin complexes and DAAC-tubulin complex were obtained from Table II.

Equilibrium constants (K_a) have been calculated using the following equation:

$$K_a = \frac{k_{on}}{k_{off}} \qquad \text{(Eq. 5)}$$

where k_{on} and k_{off} are the apparent second-order association rate constant and first-order dissociation rate constant, respectively. After calculating K_a values at different temperatures, van't Hoff plots for all four colchicinoids were done as shown in Fig. 3. Thermodynamic parameters were calculated and are presented in Table II. Our data (Table II) clearly indicate that like AC-tubulin interaction, DAAC-tubulin interaction has negative enthalpy of binding and has small positive ΔS (15.8 cal K^{-1} mol^{-1}). Both kinetic and equilibrium studies for the DAAC-tubulin interaction provide similar thermodynamic parameters.[2] However, when an amino group is substituted at C-7 in the B-ring as in NH_2-DAAC, the interaction with tubulin becomes entropy driven, and the positive ΔS increases to 46.5 cal K^{-1} mol^{-1}. The positive ΔS remains unaltered when a methyl group is substituted in NH_2-DAAC, as in demecolcine-tubulin interaction. We have reported very similar thermodynamic parameters for demecolcine-tubulin interaction using equilibrium method (31). Addition of another methyl group as in NMe_2-DAAC causes a further increase in entropy to 54.9 cal K^{-1} mol^{-1}. These data clearly establish that the bare B-ring itself has no significant effect in the thermodynamics of drug binding with tubulin. Rather, it is the B-ring substituent(s) that convert an enthalpy-driven reaction into an entropy-driven one. Early equilibrium studies on colchicine-tubulin interaction reported high positive entropy value for the binding reaction (8, 24). Later, these data were questioned for two reasons: first, these were possibly obtained in conditions where true equilibrium has not been reached; second, proper corrections were not made for the decay of colchicine binding site (20). In the study of Diaz and Andreu (25), where corrections were made for the decay of colchicine binding site, the colchine-tubulin interaction was found to be accompanied by negative enthalpy change. A negative enthalpy value for the colchicine-tubulin interaction was also obtained from calorimetric study (20). It should be noted that in one of the earlier equilibrium studies, tubulin used for the binding was in the form of vinblastine paracrystals, where tubulin is stable for several days at room temperature (24). Moreover, vinblastine paracrystals and colchicine were incubated together for 24 h at room temperature for the binding study (24, 32). Thus, it is difficult to conceive that the decay of the colchicine binding site and non-attainment of equilibrium is responsible for the above reported result (20, 25).

It is interesting to note that the thermodynamic data of the binding reaction presented in Table II show that the changes in enthalpy and entropy upon binding are compensatory. This "compensatory" effect is shown in Fig. 4, where ΔH is plotted as a function of $T\Delta S$ at 310 K. In this plot, the slope, *i.e.* $d(\Delta H)/d(T\Delta S)$, is close to 1. It is interesting to note that values of ΔH and $T\Delta S$ for the interaction of tubulin with colchicine and AC were taken from the literature (17, 24) and plotted with that of aminocolchicinoids studied here. Similar enthalpy-entropy compensation with slope close to 1 has been observed in many protein-ligand interactions where the experimental conditions are fixed and only the ligand structure is varied (Congener series) (33, 34). Arguments have been made that the perturbation, release, or shift in the state of water upon the binding of ligand to a protein is the primary source of compensating enthalpy and entropy changes. Another explanation for this compensation arises from the assumption that the protein is in equilibrium between two different states and that the ligand binds to either state with different affinities (35). Although the ligands used in this study do not induce aggregation of tubulin dimers, the effects of ligands on dimer-monomer equilibrium of tubulin may be questioned. Colchicine binding to tubulin favors dimer ⇀↽ monomer equilibrium toward dimer (36–38). We observed that all of these colchicinoids affect dimer ⇀↽ monomer equilibrium and favors dimer formation very similar to that of colchicine. Furthermore, it has now been established that colchicine and its analogs can bind tubulin in its dimer and monomer forms equally well (39, 40). Since all of these ligands affects dimer ⇀↽ monomer equilibrium similarly and in the same direction, and as both dimer and monomer of tubulin can bind ligands equally well, the differential affects of ligands on the state of association of tubulin do not arise. It was recently proposed by Pyles and Bane Hastie (22) that the B-ring substituent of aminocolchicinoids resides outside the colchicine binding site and makes contact(s) with tubulin. Results presented in this report support their hypothesis. This contact of the substituent with the protein would cause a reorganization of the water structure around the protein and the ligand species toward a greater disorder of the water molecules compared

[2] G. Chakrabarti, S. Sengupta, and B. Bhattacharyya, unpublished observation.

to the isolated individually hydrated species (41). This probably is the simple explanation for the observed high values of entropy change accompanying the binding reaction.

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REFERENCES

1. Beck, A. (1932) *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol.* **165,** 208
2. Zemer, D., Revach, M., Pras, M., Modan, B., Schor, S. & Gafin, J. (1974) *N. Engl. J. Med.* **289,** 932
3. Capraro, H. G. & Brossi, A. (1984) *The Alkaloids* (Brossi, A., ed) Vol. 23, Academic Press, New York, pp. 1–70
4. Rosner, M., Capraro, H. G., Jacobson, A. E., Alwell, L., Brossi, A., Iorio, M. A., Williams, T. H., Sik, R. H. & Chignell, C. F. (1981) *J. Med. Chem.* **24,** 257–261
5. Brossi, A., Yeh, H. J. C., Chrzanowska, M., Wolff, J., Hamel, E., Lin, C. M., Quin, F., Suffness, M. & Silverton, J. (1988) *Med. Res. Rev.* **8,** 77–94
6. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7,** 4466–4479
7. Wilson, L. (1970) *Biochemistry* **9,** 4999–5007
8. Bhattacharyya, B. & Wolff, J. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 2627–2631
9. Medrano, F. J., Andreu, J. M., Gorbunoff, M. J. & Timasheff, S. N. (1989) *Biochemistry* **28,** 5589–5599
10. Hastie, S. B. (1989) *Biochemistry* **28,** 7753–7760
11. Kang, G. J., Getahun, Z., Muzaaffar, A., Brossi, A. & Hamel, E. (1990) *J. Biol. Chem.* **265,** 10255–10259
12. Andreu, J. M., Gorbunoff, M. J., Medrano, F. J., Rossi, M. & Timasheff, S. N. (1991) *Biochemistry* **30,** 3777–3786
13. Lincoln, P., Nordh, J., Deinum, J., Angstrom, J. & Norden, B. (1991) *Biochemistry* **30,** 1179–1187
14. Staretz, M. E. & Bane Hastie, S. (1993) *J. Med. Chem.* **36,** 758–764
15. Fitzgerald, T. J. (1976) *Biochem. Pharmacol.* **25,** 1383–1387
16. Ray, K., Bhattacharyya, B. & Biswas, B. B. (1981) *J. Biol. Chem.* **256,** 6241–6244
17. Bane, S., Puett, D., MacDonald, T. L. & Williams, R. C., Jr. (1984) *J. Biol. Chem.* **259,** 7391–7398
18. Andreu, J. M., Gorbunoff, M. J. Lee, J. C. & Timasheff, S. N. (1984) *Biochemistry* **23,** 1742–1752
19. Bhattacharyya, B., Howard, R., Maity, S. N., Brossi, A., Sharma, P. N. & Wolff. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2052–2055
20. Menendez, M., Laynez, J., Medrano, F. J. & Andreu, J. M. (1989) *J. Biol. Chem.* **264,** 16367–16371
21. Pyles, E. A. & Bane Hastie, S. (1993) *J. Org. Chem.* **58,** 2751–2759
22. Pyles, E. A. & Bane Hastie, S. (1993) *Biochemistry* **32,** 2329–2336
23. Andreu, J. M. & Timasheff, S. N. (1982) *Biochemistry* **21,** 534–543
24. Bryan, J. (1972) *Biochemistry* **11,** 2611–2616
25. Diaz, J. F. & Andreu, J. M. (1991) *J. Biol. Chem.* **266,** 2890–2896
26. Hamel, E. & Lin, L. (1981) *Arch. Biochem. Biophys.* **209,** 29–40
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
28. Lambeir, A. & Engelborghs, Y. (1981) *J. Biol. Chem.* **256,** 3279–3282
29. Banerjee, A., D'Hoore, A. & Engelborghs, Y. (1994) *J. Biol. Chem.* **269,** 10324–10329
30. Barrow, G. M. (1973) *Physical Chemistry* 3rd Ed., p. 473, New York, McGraw-Hill, Inc.
31. Ray, K., Bhattacharyya, B. & Biswas, B. B. (1984) *Eur. J. Biochem.* **142,** 577–581
32. Bryan, J. (1971) *Exp. Cell. Res.* **66,** 129–136
33. Lumry, R. & Rajender, S. (1970) *Biopolymers* **9,** 2890–2896
34. Belleau, B. & Lavoie, J. L. (1988) *Can. J. Biochem.* **46,** 1397–1409
35. Eftink, M. R., Anusiem, A. C. & Biltonen, R. L. (1983) *Biochemistry* **22,** 3884–3896
36. Detrich, H. W., III, Williams, R. C., Jr. & Wilson, L. (1982) *Biochemistry* **21,** 2392–2400
37. Sackett, D. L. & Lippoldt, R. E. (1991) *Biochemistry* **30,** 3511–3517
38. Panda, D., Roy, S. & Bhattacharyya, B. (1992) *Biochemistry* **31,** 9709–9716
39. Shearwin, K. E. & Timasheff, S. N. (1994) *Biochemistry* **33,** 894–901
40. Wolff, J. & Knipling, L. (1995) *J. Biol. Chem.* **270,** 16809–16812
41. Ross, P. D. & Subramaniam, S. (1981) *Biochemistry* **20,** 3096–3102
42. Garland, D. L. (1978) *Biochemistry* **17,** 4266–4275