-NH-Dansyl Isocolchicine Exhibits a Significantly Improved Tubulin-Binding Affinity and Microtubule Inhibition in Comparison to Isocolchicine by Binding Tubulin through Its A and B Rings†

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ABSTRACT: Structure−activity relationship studies have established that the A and C rings of colchicine comprise the minimum structural feature necessary for high affinity drug−tubulin binding. Thus, colchicine acts as a bifunctional ligand by making two points of attachment to the protein. Furthermore, analogues belonging to the iso series of colchicine are virtually inactive in binding to tubulin and inhibiting microtubule assembly. In the present study, we found that the substitution of a hydrophobic dansyl group on the B-ring side chain (C7 position) of isocolchicine reverses the structural alterations at the C ring and the newly synthesized -NH-dansyl isocolchicine restores the lost biological activity of the compound. It inhibits microtubule assembly efficiently with an IC_{50} value of 10 μM and competes with [^{3}H]colchicine for binding to tubulin. Moreover, although -NH-dansyl colchicine binding to tubulin involves two steps, the -NH-dansyl isocolchicine−tubulin interaction has been found to occur via a one-step process. Also, the affinity constant of the -NH-dansyl isocolchicine−tubulin interaction is roughly only 3 times lower than that of the -NH-dansyl colchicine−tubulin interaction. These results suggest that the enhanced microtubule inhibitory ability of -NH-dansyl isocolchicine is therefore related to the affinity of the drug−tubulin interaction and not to any conformational changes upon binding tubulin. We also observed that the competition of -NH-dansyl isocolchicine with [^{3}H]colchicine for binding to tubulin was dependent on the tubulin concentration. In conclusion, this paper for the first time indicates that a biologically active bifuntional colchicine analogue can be designed where the drug binds tubulin through its A and B rings, while the C ring remains inactive.

The vinblastine- and taxol-binding sites on tubulin have successfully been targeted in the development of anticancer drugs. However, the colchicine-binding site, the most studied among antimitotic drugs, has not shown such promise until recently because of toxicity reasons. Currently, the known drugs in phase I clinical trials that bind tubulin at the colchicine site are E7010 (*N*-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide) (*1*) and combretastatin (*2*, *3*), although the structure of E7010 is unrelated to that of colchicine. Structurally, colchicine consists of a three-ringed system: a trimethoxy phenyl ring (A ring), a saturated seven-membered ring containing an acetamido group (B ring), and a tropolone ring (C ring). Attempts to design colchicine analogues that bind tubulin with high affinity have typically focused on utilizing structure−activity relationship (SAR)[1] studies. For example, colchicine analogues modified at or even depleted of the B ring retain antimitotic and antimicrotubule assembly activity (*4*). Thus, previous SAR studies of the colchicine−tubulin interaction have shown that the A and C rings of colchicine comprise the minimum structural feature necessary for high affinity drug−tubulin binding. In addition, previous binding studies clearly indicate that colchicine is a bifunctional ligand that interacts with tubulin through two parts, i.e., the trimethoxy phenyl (A ring) and tropolone (C ring) rings. The binding studies with individual ring compounds such as mescaline (a A-ring analogue) and tropolone methyl ether (a C-ring analogue) have shown that both compounds bind to tubulin with very low affinity (10^2-10^3 M^{-1}) (*5*). However, when the A and C rings are connected through a single bond forming a compound called AC [2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone], tubulin binding is 500 times tighter (10^5-10^6 M^{-1}) (*6*). As a result, it can be concluded that two points of attachment by the drug to tubulin is required for high affinity molecule binding.

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[1] Abbreviations: SAR, structure−activity relationship; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulphonic acid); EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $MgCl_2$, magnesium chloride; GTP, guanosine-5′-triphosphate; GTPase, guanosine-5′-triphosphatase; Me_2SO, dimethyl sulfoxide; PCA, perchloric acid; AC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; ΔASA, change in accessible surface area.

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Further evidence of the essential contribution of both the A and C rings toward binding affinity is clearly seen through the following studies. The substitution of one -OCH_3 group on the A ring by a hexose (colchicoside) completely abolished the ability of the drug to displace colchicine from tubulin (*7*). Similarly, lumicolchicine, which has an intact A ring but possesses a modified C ring, binds tubulin very weakly ($K_a \cong 640\ M^{-1}$) (*8*). Isocolchicine, a colchicine analogue in which the position of the C-ring methoxy and carbonyl are exchanged, is virtually inactive in binding to tubulin (*9*, *10*) and is unable to inhibit the formation of microtubule assembly (*11*). Earlier reports from our laboratory have also shown that the introduction of a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) moiety on the B ring of colcemid does not alter its tubulin-binding affinity. However, the introduction of the same NBD group on the B ring (C7 position) of inactive isocolcemid increases its tubulin-binding affinity significantly (*12*).

In the present study, we introduced a hydrophobic dansyl group on the B ring of isocolchicine (side chain at C7 position). Our results in this report show that -NH-dansyl isocolchicine (Figure 1A) can inhibit tubulin assembly efficiently and it competes with [^{3}H]colchicine for binding to tubulin. Thus, the introduction of this dansyl moiety on the B ring of isocolchicine has been shown to enhance the biological activity of the otherwise inactive isocolchicine molecule. In addition, these results indicate that a biologically active bifunctional colchicine analogue can be designed where the drug binds tubulin through its A and B rings, while the C ring remains inactive.

MATERIALS AND METHODS

Materials. Piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (PIPES), ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), guanosine-5′-triphosphate (GTP), colchicine, and podophyllotoxin were purchased from Sigma. Perchloric acid (PCA), ammonium molybdate, and malachite green were purchased from Merck. Radioactive colchicine ([^{3}H]-labeled C-ring methoxy, 60.0 Ci/mmol) was obtained from New England Nuclear. The cell line HeLa was obtained from NCCS, Pune, India, and HCT 116 was from ATCC, U.S.A. DEAE-81 filter paper was the product of Whatman. Other reagents were of analytical grade.

-NH-dansyl colchicine and -NH-dansyl isocolchicine were synthesized from deacetyl colchicine and deacetyl isocolchicine, respectively, using dansyl chloride (details of the synthesis will be published later). The purity and identity of the two compounds was determined using ^{1}H NMR and TLC analysis.

Drugs. A stock solution was made with 100% DMSO, and the drug concentration was determined from the extinction coefficient of $1.6694 \times 10^4\ M^{-1}\ cm^{-1}$ at 350 nm for -NH-dansyl colchicine and $1.6861 \times 10^4\ M^{-1}\ cm^{-1}$ at 344 nm for -NH-dansyl isocolchicine, respectively.

Tubulin Isolation and Estimation. Microtubular proteins were isolated from goat brains by two cycles of a temperature-dependent assembly−disassembly process. Pure tubulin was isolated from microtubular proteins by two additional cycles of temperature-dependent polymerization and depolymerization using 1 M glutamate buffer for assembly (*13*). The composition of the assembly buffer was 50 mM PIPES

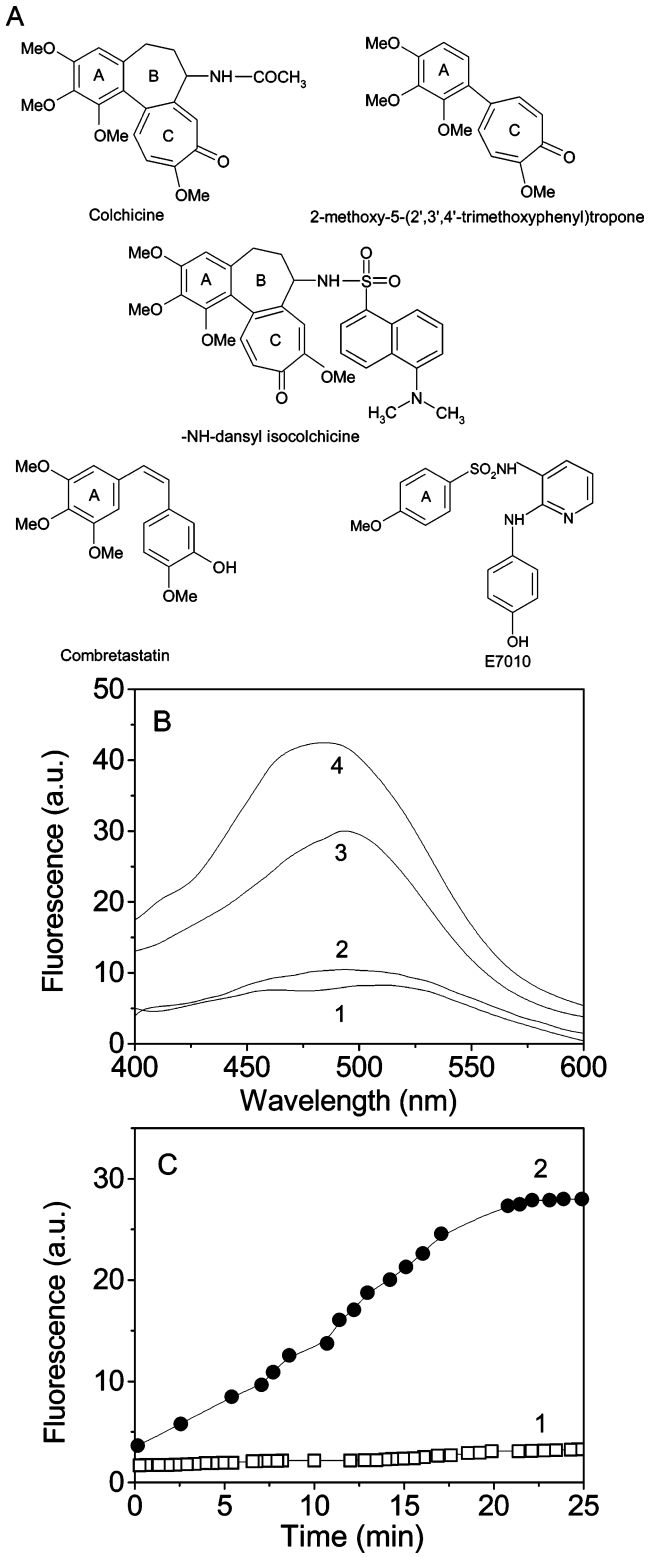

FIGURE 1: (A) Structures of colchicine, AC, -NH-dansyl isocolchicine, combretastatin, and E7010. (B) Time-dependence binding of -NH-dansyl isocolchicine with tubulin. Fluorescence emission spectra of -NH-dansyl isocolchicine only (curve 1), -NH-dansyl isocolchicine−tubulin complex at 0 min (curve 2), 15 min (curve 3), and 30 min (curve 4) incubation at 37 °C. The complex was prepared in PEM buffer by mixing 5 μM tubulin and 20 μM -NH-dansyl isocolchicine. The excitation and emission wavelengths were 344 and 490 nm, respectively. (C) Temperature-dependence binding of -NH-dansyl isocolchicine with tubulin. Tubulin (5 μM) was mixed with 20 μM -NH-dansyl isocolchicine and incubated at 4 °C (□) and 37 °C (●) for the indicated time period. The excitation and emission wavelengths were the same as in Figure 1B.

at pH 6.9, 1 mM EGTA, 0.5 mM magnesium chloride ($MgCl_2$), and 0.5 mM GTP. The protein was stored at −70 °C. The protein concentration was determined by the method of Lowry et al. (*14*) using bovine serum albumin as the standard.

Tubulin Polymerization Assay. Pure tubulin in PEM (50 mM PIPES at pH 6.9, 1 mM EGTA, and 0.5 mM $MgCl_2$) buffer was polymerized at 37 °C in the presence of 1 mM GTP. Polymerization was initiated using 10% dimethyl sulfoxide (Me_2SO), and the turbidity was measured by the absorbance at 410 nm rather than at the usual 360 nm (because -NH-dansyl colchicine and -NH-dansyl isocolchicine have absorption maxima at 350 and 344 nm, respectively). A Shimadzu UV-160 double-beam spectrophotometer, fitted with a temperature-controlled circulating water bath accurate to ±0.2 °C was used for this purpose. IC_{50} values were calculated using the concentration of the drug that caused 50% inhibition of the polymer mass.

Cytotoxicity Assay. MTT assay (*15*) was used to determine the number of viable cells upon drug addition. Cells were seeded in microtiter plates (generally 3×10^3 cells per well) and were incubated with different concentrations of the cytotoxic agents for 48 h. Subsequently, 100 μL of MTT solution (0.6 mg/mL) was added per well and incubated at 37 °C for an additional 2 h. The amount of formazan salt was quantified in quadruplicates by recording the absorbance at 570 nm using a Biorad Plate reader. Cell viabilities were calculated by dividing the absorbance values of the treated ones by that of the control. The drug concentrations that inhibited cell growth by 50% of the control (GI_{50}) were calculated from the semilogarithmic dose response plots using the nonlinear regression program Origin. All of the experiments were repeated at least 3 times.

[^{3}H]Colchicine-Binding Assay. The [^{3}H]colchicine-binding assay was performed by the filter disk method using DEAE-81 filter paper (*16*).

Binding Measurements by the Fluorescence Method. The binding of the ligands to the protein was monitored by enhancement of ligand fluorescence in the presence of protein. Fluorescence spectra were recorded using a Hitachi F-3000 fluorescence spectrophotometer connected to a constant temperature circulating water bath accurate to ±0.2 °C. All fluorescence measurements were carried out in a 0.5 cm path-length quartz cuvette, and fluorescence values were corrected for the inner-filter effect using the following equation of Lakowicz (*17*):

$$F_{cor} = F_{obs}\,\{\text{anti log}(A_{ex} + A_{em})/2\}$$

where A_{ex} and A_{em} are the absorbance at the excitation and the emission wavelength, respectively.

Association Rate Constant. The association rate constant was determined from measurements of the initial rate of the dansyl analogues binding to tubulin. If drug binding to tubulin is assumed to be bimolecular, then the association rate constant (k_1) is,

$$k_1 = \frac{\mathrm{d}[DT]}{\mathrm{d}t}\Big/[D][T]$$

where [*D*] and [*T*] are the concentrations of free drug and unoccupied tubulin, respectively. d[*DT*]/d*t* is the rate of formation of the complex (dansyl analogue−tubulin). Conditions were adjusted such that <10% of the reactants were consumed during the reaction, and the progress curves were linear. The amount of dansyl analogue-bound to the protein was determined by fluorescence as follows: we constructed a standard curve (for each drug) by titrating 3 μM drug with increasing amounts of tubulin (up to 30 μM) until the corrected fluorescence because the bound ligand had reached saturation. From this curve, the fluorescence intensities could be used to calculate the amount of ligand-bound tubulin. The excitation wavelengths were 350 nm for -NH-dansyl colchicine and 344 nm for -NH-dansyl isocolchicine, and the emission wavelengths were 490 nm for -NH-dansyl isocolchicine and 495 nm for -NH-dansyl colchicine.

To determine the rate constant, the enhancement of fluorescence of the ligand as a function of time was plotted. From the slope of the initial rate, the rate of formation of the complex was calculated. Tubulin was held constant at 3 μM for -NH-dansyl colchicine and at 2 μM for -NH-dansyl isocolchicine (Table 2). The drug concentration was varied from 20 to 100 μM. The measurement of the fluorescence was done at 490 nm for -NH-dansyl isocolchicine and 495 nm for -NH-dansyl colchicine, respectively, upon excitation of the samples at 350 nm for -NH-dansyl colchicine and 344 nm for -NH-dansyl isocolchicine, respectively.

Activation Energy. The association rate constant (k_1) was determined at different temperatures ranging from 30 to 37 °C, and the activation energy (E_a) was calculated by plotting ln k_1 against $1/T$ according to the Arrhenius equation, $k_1 = A \exp(-E_a/RT)$, where *A* is the pre-exponential factor.

Dissociation Kinetics. The dissociation of the -NH-dansyl colchicine−tubulin complex was 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 (*6*) in the presence of 2 M glycerol. This process was described as a single first-order reaction. The rate constant of this process was determined using the relation

$$\ln(F_{max} - F_t) = k_{off}t + \text{constant}$$

where F_{max} and F_t are the maximum intrinsic protein fluorescence intensity at infinite time and time *t*, respectively, and k_{off} is the first-order dissociation rate constant. The dissociation rate constant is determined at 37 °C.

Scatchard Analysis. We determined the affinity constant and stoichiometry for both -NH-dansyl isocolchicine and -NH-dansyl colchicine with tubulin using a conventional Scatchard analysis (*18*)

$$\frac{r}{D} = nK - Kr \qquad (1)$$

where *r* is the number of moles of drug bound per mole of tubulin, *D* is the free drug concentration, *K* is the affinity constant, and *n* is the number of drug-binding sites on tubulin. We performed a reverse titration using 3 μM of the drug with an increasing concentration of tubulin, and a standard curve was obtained when 1/fluorescence was plotted against 1/[protein]. From this curve, the fluorescence intensity corresponding to the 3 μM ligand−tubulin complex was determined. To generate the binding isotherm, the dansyl

analogue (2−24 μM) was added to 3 μM tubulin and each sample was incubated at 37 °C for 60 min. The amount of bound drug was determined by measuring the fluorescence at 490 nm for -NH-dansyl isocolchicine and 495 nm for -NH-dansyl colchicine, respectively, upon excitation of the samples at 350 nm for -NH-dansyl colchicine and 344 nm for -NH-dansyl isocolchicine, respectively. Because the bound drug concentration was known from the standard curve, the free drug concentration was calculated from the total drug concentration. We plotted r/D against r, and the affinity constant of the drug was calculated from the slope of the plot.

Guanosine-5′-triphosphatase (GTPase) Assay. The effect of various colchicine analogues on tubulin-mediated hydrolysis of GTP was assessed using a malachite green sodium molybdate assay (*19*, *20*). Separate tubulin samples (10 μM) were prepared in PEM buffer with 100 μM colchicine analogues. The samples were incubated at 37 °C for 60 min. Subsequently, 0.1 mM GTP was added to each sample, and the solutions were again incubated at 37 °C. Aliquots (70 μL) were withdrawn at different time intervals, and the reaction was quenched with 7 μL of 10% v/v PCA. Samples were centrifuged, and 50 μL of the supernatant was added to 950 μL of a mixture of 0.13% malachite green, 4.2% ammonium molybdate, and 0.02% Triton X-100 in 6 N H_2SO_4 and then kept in the dark for 1 h. The GTPase activity was determined by measuring the absorbance at 630 nm. The phosphate content of the unknown samples was determined from the sodium phosphate standard curve.

Modeling of the Tubulin−Drug Complex. The model of the tubulin−drug complex was generated from the crystal structure of the colchicine-bound−tubulin complexed with stathmin-like domain (PDB ID 1SA0) (*21*). The coordinates corresponding to one tubulin dimer bound to colchicine were removed. The colchicine molecule was replaced with the -NH-dansyl colchicine or -NH-dansyl isocolchicine. Coordinates corresponding to the dansyl group were obtained from the Hic Up server (http://alpha2.bmc.uu.se/hicup/) (*22*) and were linked to the colchicine moiety using DISCOVER (Biosym/MSI). The resultant modeled complex was subjected to energy minimization using the steepest decent method followed by conjugate gradient in the BIOSYM module of INSIGHT II. Default parameters were used in both minimization processes.

RESULTS

Binding of -NH-Dansyl Isocolchicine to Tubulin. The binding of -NH-dansyl isocolchicine to tubulin was studied using the fluorescence of the dansyl group. Binding to tubulin enhanced the -NH-dansyl isocolchicine fluorescence intensity with a large blue shift of the emission maxima. The emission maximum of free -NH-dansyl isocolchicine shifted 20 nm towards the blue upon binding to tubulin (λ_{max} shifted from 510 to 490 nm). This enhancement of fluorescence intensity and the incumbent shift of λ_{max} changed with the time of incubation of -NH-dansyl isocolchicine and tubulin at 37 °C (shown in Figure 1B). In addition, the observed binding of -NH-dansyl isocolchicine was not solely due to the presence of the dansyl group, because the dansyl compound minus a colchicine moiety did not bind to tubulin (Table 1). Therefore, these results indicate that the binding (and the subsequent effect of enhancement of fluorescence and the shifting of λ_{max}) of -NH-dansyl isocolchicine to tubulin is due to the combined effect of both the dansyl group and the isocolchicine nucleus.

Table 1: Fluorescence of the Dansyl Derivatives in the Presence and Absence of Tubulin[a]

dansyl derivative	fluorescence without tubulin (a.u.)	fluorescence with tubulin (a.u.)
-NH-dansyl colchicine	5	19.3
-NH-dansyl isocolchicine	5.2	16.2
dansyl-2-mercaptoethanol	25	24

[a] Tubulin (3 μM) was mixed with different dansyl derivatives (10 μM) in PEM buffer at 37 °C. Fluorescence was measured after incubation for 30 min at 37 °C.

The temperature dependence of the aforementioned binding is shown in Figure 1C. This binding between tubulin (4 μM) and -NH-dansyl isocolchicine (20 μM) was performed at 37 °C (curve 2) and 4 °C (curve 1). As can be seen in Figure 1C, it is very clear that the binding is highly temperature-dependent with virtually no time-dependent binding at 4 °C.

To determine where -NH-dansyl isocolchicine binds to tubulin, the time dependence binding of -NH-dansyl isocolchicine to both tubulin and the tubulin−colchicine complex was tested. This was done through the observation of its fluorescence as a function of time. Curve 2 of Figure 2A shows the binding of 5 μM tubulin and 20 μM -NH-dansyl isocolchicine at 37 °C. Also, curve 1 (Figure 2A) shows that -NH-dansyl isocolchicine does not bind to tubulin when the colchicine-binding site is occupied by either colchicine or any colchicine analogues such as podophyllotoxin or nocodazole (data not shown). Thus, to determine whether the binding occurs at the colchicine site, -NH-dansyl isocolchicine was allowed to compete with podophyllotoxin for binding to tubulin and the data were analyzed using a modified Dixon plot (Figure 2B). The results presented in Figure 2B clearly indicate that podophyllotoxin binding was inhibited competitively by -NH-dansyl isocolchicine yielding an apparent K_i value of about 7.3 μM. Also, as shown in Figure 2C, -NH-dansyl isocolchicine competes for the [^{3}H]colchicine-binding site on tubulin. In this experiment, a fixed concentration of tubulin was allowed to bind a mixture containing a fixed concentration of [^{3}H]colchicine and a variable concentration of -NH-dansyl isocolchicine (Figure 2C). It was observed that nearly 40% inhibition of [^{3}H]colchicine binding occurred at 100 μM -NH-dansyl isocolchicine (curve 1). Under identical conditions, 100 μM isocolchicine caused hardly any detectable inhibition of [^{3}H]colchicine binding (curve 2).

Kinetic Parameters. Association rate constants were measured assuming that drug bound to tubulin is a bimolecular reaction. In our experiment, drug concentration was varied while keeping the protein concentration fixed. The second-order rate constants (k_1) calculated are listed in Table 2. The mean k_1 was 2.81×10^4 M^{-1} h^{-1} for -NH-dansyl isocolchicine (k_1 for -NH-dansyl colchicine was 8.73×10^4 M^{-1} h^{-1}) at 37 °C. This is almost 10 times lower than that of colchicine (*23*). We determined k_1 at four different temperatures (30, 32, 34, and 37 °C) and thereby calculated the activation energy for -NH-dansyl isocolchicine binding to tubulin. Because the reaction rate was very slow below

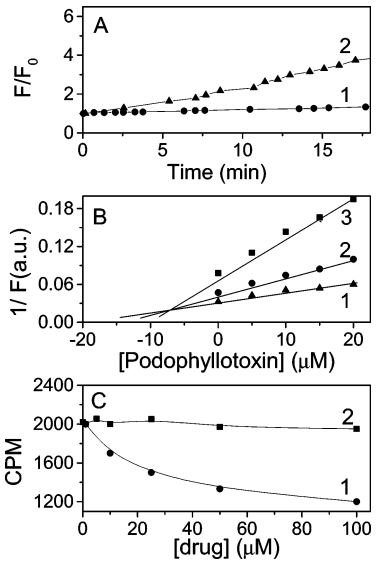

FIGURE 2: -NH-dansyl isocolchicine binding to the colchicine-binding site of tubulin. (A) Binding of 20 μM -NH-dansyl isocolchicine to 4 μM tubulin (▲), and the tubulin−colchicine complex (●). The tubulin−colchicine complex was prepared by incubating 4 μM tubulin and 20 μM colchicine at 37 °C for 60 min. The excitation and emission wavelengths were the same as in Figure 1B. (B) Modified Dixon plot. The concentrations of -NH-dansyl isocolchicine were 5 μM (■), 15 μM (●), and 30 μM (▲). The reaction mixtures contained 3 μM tubulin, -NH-dansyl isocolchicine, and podophyllotoxin at the indicated concentrations and were incubated at 37 °C for 60 min. Excitation and emission wavelengths were the same as in Figure 1B. (C) Competition of -NH-dansyl isocolchicine, isocolchicine, and [^{3}H]colchicine for the colchicine-binding site of tubulin. Tubulin (5 μM) was added to a reaction mixture containing 2 μM [^{3}H]colchicine and a varying concentration of either isocolchicine (curve 2) or -NH-dansyl isocolchicine (curve 1) in PM (10 mM sodium phosphate at pH 7.0 and 10 mM $MgCl_2$) buffer. The concentration of -NH-dansyl isocolchicine and isocolchicine are indicated in the abscissa. The reaction mixture was incubated for 1 h at 37 °C, and an assay was performed using the DEAE-81 filter disk method (*16*).

Table 2: Association Rate Constants Determined at the Different Drug Concentrations at 37 °C

[-NH-dansyl isocolchicine] (μM)	[tubulin] (μM)	rate constant (10^4 M^{-1} h^{-1})
20	2	1.64
40	2	1.86
60	2	2.08
80	2	3.50
100	2	4.95
[-NH-dansyl colchicine] (μM)	**[tubulin] (μM)**	**rate constant (10^4 M^{-1} h^{-1})**
30	3	6.39
45	3	7.59
60	3	8.91
80	3	9.74
100	3	11.01

30 °C, the rate constants were measured above 30 °C. The activation energy thus obtained for -NH-dansyl isocolchicine was 30.06 ± 0.14 kcal/mol (Figure 3). Under identical conditions, the activation energy for the -NH-dansyl colchicine was 24.11 ± 0.39 kcal/mol. These high activation energies partially explain the lower values of the association rate constants.

The dissociation of the -NH-dansyl colchicine−tubulin complex was 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 in the presence of 2 M glycerol. The off-rate constant (k_{-2}) was found to be 8.46×10^{-5} s^{-1} (figure not shown).

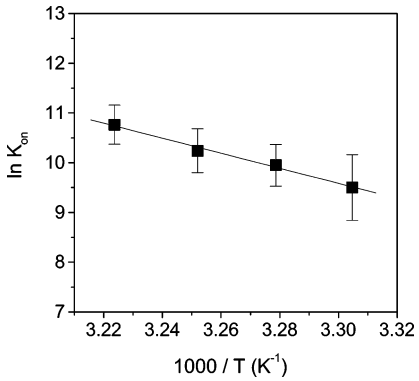

FIGURE 3: Effect of temperature on the association rate constant of -NH-dansyl isocolchicine binding to tubulin. Details of the experiment were given in the Materials and Methods.

Rate Constant and the Dependence of k_{obs} on the Reactant Concentration. It is well established that colchicine and its analogues bind to tubulin via a two-step mechanism. The initial fast step involves the formation of a low-affinity complex (TD), followed by a slow drug-induced conformational change in tubulin, resulting in formation of the stable fluorescent complex (TD)* (*6*, *24*−*26*),

$$T + D \overset{K_1}{\rightleftharpoons} TD \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (TD)^* \quad (1)$$

where K_1 is the equilibrium constant for the fast step and k_2 and k_{-2} are the on- and off-rate constants, respectively, for the second step.

The rate constant for the two-step scheme presented above under pseudo-first order conditions is given by the following hyperbolic expression (*25*):

$$k_{obs} = \frac{K_1 k_2 [D]}{1 + K_1 [D]} + k_{-2} \quad (2)$$

The above hyperbolic expression (eq 2) can be rearranged as follows:

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

Thus, a plot of $(k_{obs} - k_{-2})^{-1}$ versus $1/[D]$ will be linear with $1/(K_1 k_2)$ as the slope and $1/k_2$ as the intercept on the ordinate.

When experiments are performed with $[D]_0 \gg [T]_0$ (i.e., under pseudo-first order conditions), eq 2 would yield a similar k_{obs} expression as that described by eq 4 with $[D]_0$ as the variable (*25*),

$$k_{obs} = K_1 k_2 [D]_0 + k_{-2} \quad (4)$$

As a result, k_{obs} exhibits a nonlinear dependence on both the drug and protein concentrations (*25*). The two-step mechanism of the colchicine−tubulin interaction is based on this observation. When the dependence of k_{obs} on the ligand concentration was determined for both -NH-dansyl

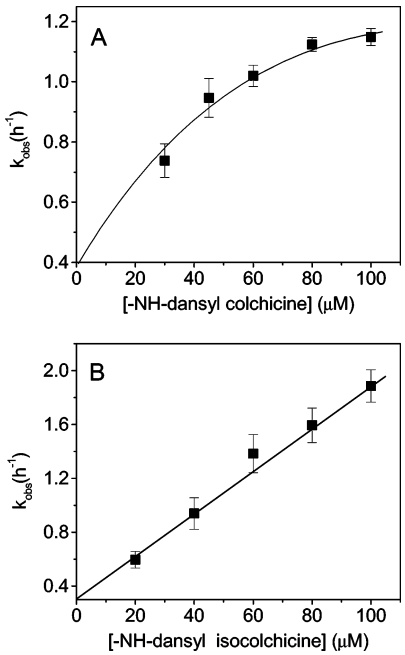

FIGURE 4: Dependence of the observed rate constant (k_{obs}) on the drug concentration. (A) k_{obs} for the tubulin−NH-dansyl colchicine reaction was plotted against the -NH-dansyl colchicine concentration. (B) k_{obs} for the tubulin−NH-dansyl isocolchicine reaction was plotted against the -NH-dansyl isocolchicine concentration. Each point represents the mean of six experiments. The tubulin concentration was 3 and 2 μM for -NH-dansyl colchicine (A) and -NH-dansyl isocolchicine (B), respectively. Excitation and emission wavelengths were 350 and 495 nm respectively for -NH-dansyl colchicine and 344 and 490 nm respectively for -NH-dansyl isocolchicine.

isocolchicine and -NH-dansyl colchicine, we found that it is a nonlinear function of the drug concentration for -NH-dansyl colchicine (Figure 4A), which is similar to that for the tubulin−colchicine interaction. Interestingly however, for the tubulin−NH-dansyl isocolchicine interaction, the k_{obs} is a linear function of the drug concentration (Figure 4B). These results suggest that, while the -NH-dansyl colchicine−tubulin binding is a two-step process, the -NH-dansyl isocolchicine−tubulin interaction is a one-step process. Thus, the reaction of these two drugs with tubulin can be represented as

$$\text{(1) tubulin} + \text{-NH-dansyl colchicine} \xrightarrow[\text{(preassociation)}]{\text{step 1}} [\text{tubulin}-\text{NH-dansyl colchicine}]$$

$$[\text{tubulin}-\text{NH-dansyl colchicine}] \xrightarrow[\text{(conformational change)}]{\text{step 2}} \text{tubulin}-\text{NH-dansyl colchicine}$$

$$\text{(2) tubulin} + \text{-NH-dansyl isocolchicine} \xrightarrow{\text{single step}} \text{tubulin}-\text{NH-dansyl isocolchicine}$$

Nearly all colchicine analogues studied so far have exhibited a two-step binding reaction with tubulin (*6*, *23*, *25*−*27*). However, recently, a very similar single-step binding process was observed and reported from this laboratory for NBD−isocolcemid binding to tubulin (*12*). This one-step binding mechanism had also been suggested for the isocolchicine−tubulin interaction based on the structural analysis of isocolchicine conformers (*9*). However, our conclusion

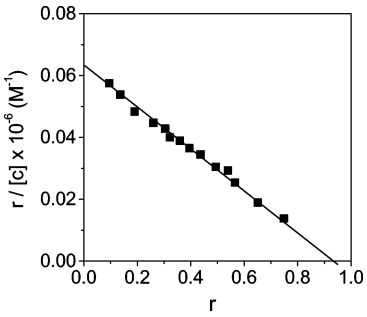

FIGURE 5: Scatchard analysis for the binding of -NH-dansyl isocolchicine to tubulin. The stoichiometry and affinity constant of -NH-dansyl isocolchicine were determined by incubating aliquots of 3 μM tubulin with 2−24 μM of the dansyl analogue at 37 °C in separate test tubes for 60 min. The amount of bound drug was then determined by measuring the fluorescence at 490 nm for -NH-dansyl isocolchicine upon excitation of the samples at 344 nm. The affinity constant was calculated from the slope of the plot. All measurements were corrected for the inner-filter effect.

regarding the one-step binding mechanism for -NH-dansyl isocolchicine is based on kinetic data.

Using eq 3, we found that the plot of $(k_{obs} - k_{-2})^{-1}$ versus 1/[*D*] for the -NH-dansyl colchicine−tubulin interaction was linear with $1/(K_1k_2)$ as the slope and $1/k_2$ as the intercept on the ordinate (figure not shown). K_1 and k_2 were evaluated and are shown in Table 3.

We determined the stoichiometry and affinity constant for both -NH-dansyl colchicine and -NH-dansyl isocolchicine with tubulin using the Scatchard analysis. The affinity constant at 37 °C for -NH-dansyl isocolchicine binding to tubulin was 0.7×10^5 M^{-1} (Figure 5), whereas for -NH-dansyl colchicine, it was 2.2×10^5 M^{-1} (data not shown). However, the affinity constant for -NH-dansyl colchicine as calculated from the kinetic parameter was 0.68×10^5 M^{-1} (Table 3). At present, the reason for the differences in the value of the equilibrium constant as calculated from these two different methods is not known.

Thus, when compared with colchicine, we observed that the substitution of a bulky dansyl group at the C7 position lowers the on rate, enhances the activation energy, and lowers the affinity constant when the drug binds with tubulin (*24*, *28*). However, in comparison with the biologically inactive isocolchicine, we observed the opposite effect. Thus, the substitution of the dansyl group at the C7 position of isocolchicine enhanced the affinity of the drug approximately 20−30 times (*9*).

Conformational Changes of Tubulin and Dansyl Analogues upon Their Interaction. The kinetic data previously presented indicates that -NH-dansyl isocolchicine binding to tubulin is a one-step reaction. Colchicine binding to tubulin however is composed of two steps. The first step involves a rapid equilibrium of the protein and the drug, which forms a low-affinity complex. This is then followed by a slow, essentially irreversible process, which forms a fluorescent complex. The conversion of the low-affinity complex to the fluorescent complex is involved in the conformational change of tubulin as well as colchicine. Substantial data support this proposal (*6*, *23*, *25*, *26*). With that in mind, we tested whether tubulin and both dansyl analogues underwent this conformational adjustment upon their interaction.

It is known that colchicine and other colchicine-site ligands stimulate GTPase activity of tubulin. This enhanced enzy-

Table 3: Kinetic Constants for a Two-Step Scheme for the -NH-Dansyl Colchicine−Tubulin Interaction

	K_1 (M^{-1})	k_2 (S^{-1})	k_{-2} (S^{-1})	[a]K_a (cal M^{-1})	[b]K_a (Scatchard) (M^{-1})
-NH-dansyl colchicine	12.77×10^3	4.499×10^{-4}	8.46×10^{-5}	0.68×10^5	2.2×10^5
[c]colchicine	$3.8-6 \times 10^3$	$1.9-2.8 \times 10^{-2}$	$5.2-9.3 \times 10^{-6}$	$1.1-2.3 \times 10^7$	2×10^6

[a] K_a was calculated for the computed kinetic constant, $K_a = K_1k_2/k_{-2}$. [b] K_a was determined from a Scatchard plot. [c] Obtained from ref *25*.

Table 4: Cytotoxic Activity of Colchicine Analogues against Different Tumor Cell Lines[a]

cell lines (source)	colchicine (μM)	-NH-dansyl colchicine (μM)	-NH-dansyl isocolchicine (μM)
HCT 116 (human colon)	0.03 ± 0.011	0.38 ± 0.1	3.12 ± 0.6
HeLa (human cervix)	0.024 ± 0.011	1.03 ± 0.3	4.12 ± 0.95

[a] Different concentrations of colchicine, -NH-dansyl colchicine, and -NH-dansyl isocolchicine were incubated at 37 °C with the different cell lines. After 48 h, drug-containing media were removed and an MTT assay was done as described in the Materials and Methods. GI_{50} values were calculated using the nonlinear regression program Origin. The average of at least three experiments is shown.

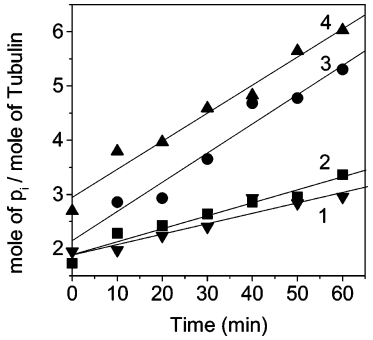

FIGURE 6: Effect of different colchicine analogues on the GTPase activity of tubulin. GTP hydrolysis by tubulin was studied in the absence (■) and presence of colchicine (●), -NH-dansyl colchicine (▲), and -NH-dansyl isocolchicine (▼). Tubulin (10 μM) was preincubated without or with each ligand (100 μM) separately for 60 min at 37 °C. Then, 10 mM GTP was added, and the incubation was continued. At various time intervals, 70 μL aliquots were withdrawn and GTP hydrolysis was assayed as described in the Materials and Methods.

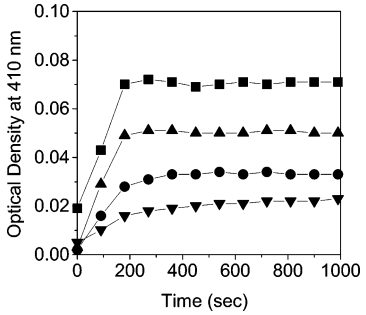

FIGURE 7: Effect of -NH-dansyl isocolchicine on tubulin polymerization. Tubulin (15 μM) was preincubated with -NH-dansyl isocolchicine at different concentrations as follows: 0 (■), 5 (▲), 10 (●), 15 (▼) μM at 37 °C for 30 min in PEM buffer. Polymerization was initiated by the addition of 10% Me_2SO followed by the addition of 1 mM GTP.

matic activity of liganded tubulin is a consequence of the drug-induced conformational changes on tubulin (*27*, *29*, *30*). Thus, we sought to determine the GTPase activity when -NH-dansyl colchicine or -NH-dansyl isocolchicine is bound to tubulin. Results of such an experiment are shown in Figure 6. The hydrolysis of GTP by tubulin is enhanced by colchicine (curve 3) and enhanced further by -NH-dansyl colchicine (curve 4). However, -NH-dansyl isocolchicine binding (curve 1) has no such effect on the GTPase activity of tubulin (curve 2 shows the GTPase activity of tubulin only).

Inhibition of Tubulin Polymerization by Dansyl Analogues. Colchicine and isocolchicine were reported to inhibit tubulin self-assembly with an IC_{50} of about 6 and 1000 μM, respectively (*9*). We tested the inhibition of tubulin polymerization of these dansyl analogues using Me_2SO. *In vitro*, both -NH-dansyl colchicine and -NH-dansyl isocolchicine show progressive concentration-dependent inhibition of tubulin self-assembly with an IC_{50} of 7.1 and 10 μM, respectively (Figure 7).

To extrapolate the microtubule inhibition activities in the *in vivo* condition, we investigated the effect of -NH-dansyl colchicine and -NH-dansyl isocolchicine on tumor cell growth. Tumor cells from human cervix and colon were treated with different concentrations of the drugs. For comparison, colchicine was also used. Cytotoxicity was measured by the cellular metabolic activity using the MTT assay. The results are summarized in Table 4. In HeLa and HCT 116 cell lines, as compared by the GI_{50} values, -NH-dansyl isocolchicine is roughly 4 and 8 times less active than -NH-dansyl colchicine, respectively.

DISCUSSION

The analogues belonging to the iso series of colchicine (the relative positions of -OCH_3 and >C═O group in the C ring are interchanged as compared to colchicine) are believed to be inactive in binding to tubulin (*9*, *10*, *24*). In contrast, this study shows that -NH-dansyl isocolchicine can bind to tubulin with high affinity. The remarkable observations from this study can be summarized in the following points: (i) The affinity constant of the -NH-dansyl isocolchicine−tubulin interaction is 0.7×10^5 M^{-1}, which is roughly 3 times lower than that of the -NH-dansyl colchicine−tubulin interaction. In contrast, the affinity constant of isocolchicine for the colchicine receptor site, measured using different techniques, is varied from 600 to 4.4×10^3 M^{-1} (*9*, *10*). Thus, the affinity constant of the isocolchicine−tubulin interaction is about 500 times lower than that of the colchicine−tubulin interaction (*24*, *32*, *33*). (ii) -NH-dansyl isocolchicine competes with [^{3}H]colchicine for binding to tubulin (Figure 2C). (iii) This drug also inhibits tubulin polymerization efficiently with an IC_{50} value of 10 μM (Figure 7). (iv) -NH-dansyl-isocolchicine upon binding to tubulin does not enhance the GTPase activity of the latter. Thus, it seems that the conformational changes of tubulin and the drug that occur during colchicine binding do not take place in the case of the iso derivative. This inference has also been substantiated by kinetic data, where we

observed the linear dependence of k_{obs} with an increasing concentration of -NH-dansyl isocolchicine and a nonlinear dependence of k_{obs} with an increasing concentration of -NH-dansyl colchicine (same as colchicine) (Figure 4). Therefore, this kinetic data indicates that, like the colchicine−tubulin interaction, the binding of -NH-dansyl colchicine to tubulin is composed of two steps, while -NH-dansyl isocolchicine binding to tubulin is a one-step process.

Isocolchicine has two low-affinity sites on tubulin. The molecule binds rapidly to the first site, competing with AC, and its behavior resembles that of A-ring analogues (*10*). The second site however, is not well-characterized, but it does not overlap the AC-′′′binding site (*10*). In addition, it is also known that isocolchicine binds to tubulin in one step in a reversible manner (*9*). Therefore, the molecule does not undergo the second binding step that is responsible for the conformational changes that occur in the drug and tubulin upon their interaction. -NH-Dansyl isocolchicine is an interesting colchicine analogue that sheds additional light on this aspect. Similar to isocolchicine, -NH-dansyl isocolchicine binding to tubulin has been shown to occur in one step and is thus not followed by the second step involving conformational changes. Therefore, this would clearly suggest that the microtubule inhibitory ability of -NH-dansyl isocolchicine is probably not related to these conformational changes. Rather, it indicates that the affinity of the drug−tubulin interaction is the determining factor in the potency of the drug of inhibition. This is also supported by the fact that isocolchicine, which does not undergo the second step, is also an inhibitor (although very poor) because of its lower tubulin-binding affinity (*9*).

With that in mind, how then does -NH-dansyl isocolchicine enhance its binding affinity towards tubulin in comparison to that of isocolchicine? It seems justified to assume that the altered C ring of -NH-dansyl isocolchicine does not contribute towards binding affinity and the increase in the affinity must be a consequence of the dansyl substitution at the C7 position. This substitution would allow -NH-dansyl isocolchicine to act as a bifunctional ligand, by making two points of attachment to tubulin through its A- and B-ring side chain. The interaction of the latter, i.e., the B-ring side chain, with α tubulin had been hypothesized long ago (*23*, *28*, *34*). In the present study, we have shown that the binding of tubulin to -NH-dansyl colchicine and -NH-dansyl isocolchicine very significantly shifted the emission maxima of the respective drugs towards the blue (Figure 1B). These results indicate that the dansyl group does get buried upon tubulin binding.

The above conclusion gains further support from the observation that the competition of -NH-dansyl isocolchicine with [^{3}H]colchicine for binding to tubulin is dependent on the tubulin concentration (Figure 8). At a very low concentration of protein (0.3 μM), where tubulin predominantly exists in the monomeric state (*35*), -NH-dansyl isocolchicine competes poorly with [^{3}H]colchicine for binding to the protein. This is a consequence of α and β tubulin being separated, which results in -NH-dansyl isocolchicine binding only β tubulin with a very low affinity through its A ring only. In this case, the B ring containing the dansyl moiety remains free and exposed to the solvent. As a result, the B-ring side chain of the drug molecule would then be unable to contribute towards its binding affinity. Figure 9 summarizes the possible explanation of the above result (Figure 8) in the form of a model.

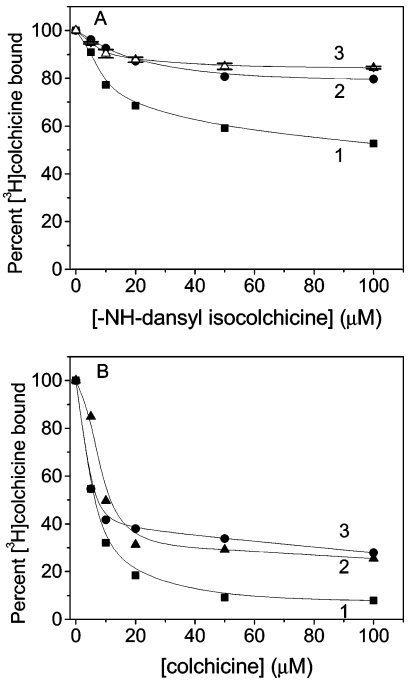

FIGURE 8: Competition of colchicine, -NH-dansyl isocolchicine, and [^{3}H]colchicine for binding to tubulin at its various concentrations. (A) Reaction mixtures containing 3 μM [^{3}H]colchicine and various concentrations of -NH-dansyl isocolchicine were allowed to bind with 2 μM (curve 1), 1 μM (curve 2), and 0.3 μM (curve 3) tubulin at 37 °C for 1 h. The points corresponding to 0.3 μM tubulin represent the mean of three experiments. The -NH-dansyl isocolchicine concentration is indicated in the abscissa. Bound [^{3}H]-colchicine in the absence of -NH-dansyl isocolchicine has been taken as 100%. (B) Reaction mixtures containing 3 μM [^{3}H]colchicine and various concentrations of colchicine were allowed to bind with 2 μM (curve 1), 1 μM (curve 2), and 0.3 μM (curve 3) tubulin at 37 °C for 1 h. Colchicine concentration is indicated in the abscissa. Bound [^{3}H]colchicine in the absence of colchicine has been taken as 100%.

A molecular-modeling exercise based on the recently determined crystal structure of the tubulin−colchicine complex (*21*) further strengthened the above proposition concerning the dansyl group−tubulin interaction. For example, replacing colchicine with -NH-dansyl colchicine or -NH-dansyl isocolchicine in the complex shows that the dansyl group gets buried deep inside the α subunit of tubulin (Figure 10), leading to a large change in the accessible surface area (ΔASA) of the drug upon binding (ΔASA for -NH-dansyl isocolchicine and -NH-dansyl colchicine are 792.3 and 827.3 Å^2, respectively). Such ΔASA values explain the significant blue shifting in the emission maxima of the hydrophobic dansyl groups. The crystal structure enabled us to look at the molecular details of the tubulin−colchicine interaction. In agreement with the biochemical observations, the structure shows that the A ring of the drug interacts with β tubulin, while the B-ring side chain interacts with the α chain. On the other hand, the C ring seems to interact with both chains through the formation of hydrogen bonds via the >C═O moiety (with peptide-NH of valine 181 and γ-NH_2 of lysine 352 from α and β tubulin, respectively) (Figure 11). Thus, it seems obvious that an interchange of the >C═O and -OCH_3 groups as in isocolchicine would affect these hydrogen bonds apart from steric constraints. This interaction

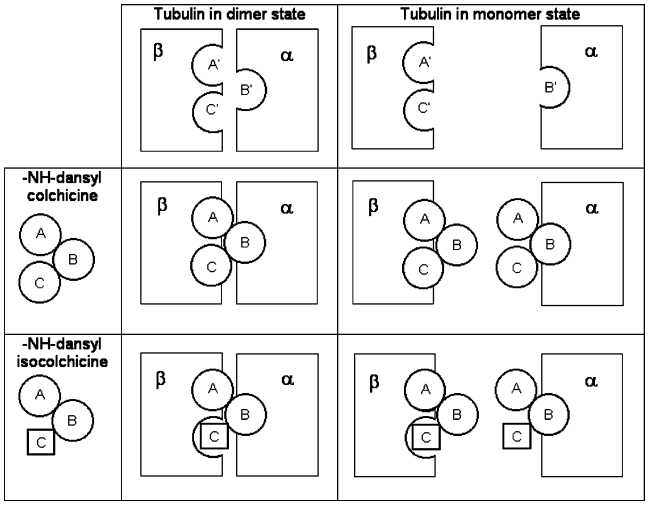

FIGURE 9: Schematic representation of the interaction of -NH-dansyl colchicine and -NH-dansyl isocolchicine with tubulin in dimeric and monomeric conditions. The α subunit has one binding site for the B-ring side chain of the colchicine analogue and is labeled as B′. The *β* subunit has two binding sites for the A and C rings and is labeled as A′ and C′, respectively. The compound -NH-dansyl colchicine is represented as a combination of three circles A, B, and C, whereas -NH-dansyl isocolchicine is represented as a combination of two circles (for A and B rings) and one box (C ring) because this C ring does not contribute to the binding affinity. In the case of -NH-dansyl colchicine, in the dimeric state, A, B, and C rings contribute towards the binding affinity, whereas in the monomeric state, binding occurs through either the A and C rings or the B ring. In case of -NH-dansyl isocolchicine, in the dimeric state, binding occurs through both the A and B rings. However, in the monomeric condition, binding only occurs through an individual ring, by either the A or B ring.

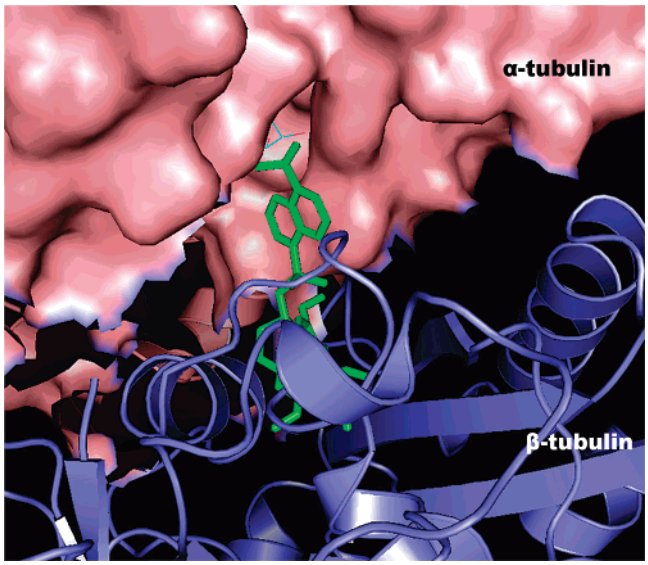

FIGURE 10: Model structure of the -NH-dansyl isocolchicine−tubulin complex showing the α tubulin (surface representation in salmon) and *β* tubulin (cartoon representation in slate). The drug molecule viewed in "sticks" can be observed to have its dansyl moiety buried deep inside the α tubulin. The model has been generated from the tubulin−colchicine complex crystal structure (*21*) (PDB ID 1SA0) using DISCOVER (Biosym/MSI).

of the C ring with α tubulin was previously unknown. We have also used the models of the -NH-dansyl colchicine− and -NH-dansyl isocolchicine−tubulin complex to gain insights into the differences regarding the interaction of the two drugs with the protein. The result, although not providing a detailed understanding, does show significant variation between the structures of the two complexes (Figure 12). From this figure, the two drugs can be seen to have different orientations of their colchicine moiety, while the dansyl moieties occupy almost the same position. Moreover, the conformations of α tubulin are not significantly different in these complexes (rmsd < 0.1 Å), but large variations in the respective *β* subunits are noteworthy (rmsd ∼ 0.9 Å). These conformational differences in the protein and the drug may account for the difference in the biochemi-

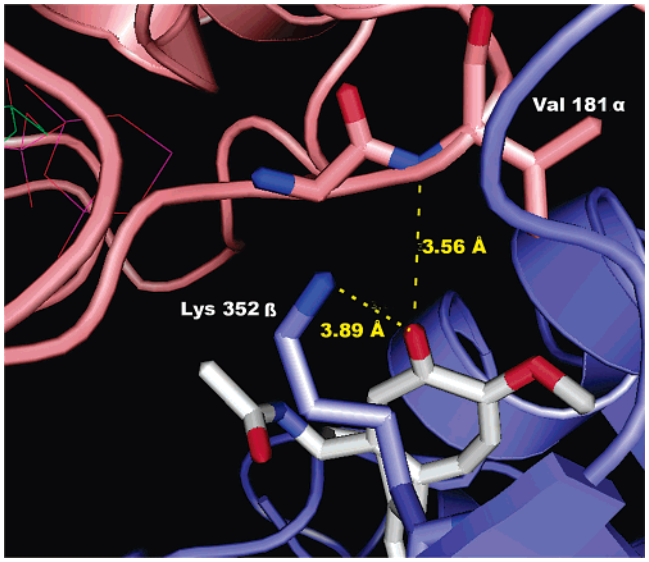

FIGURE 11: Interaction of the C-ring carbonyl group of the colchicine molecule with tubulin. The probable hydrogen bonds via the >C═O moiety (with peptide-NH of valine 181 and γ-NH_2 of lysine 352 from α and *β* tubulin, respectively) have been indicated using a dotted line along with the bond distance. This figure has been generated using the software PYMOL (*36*) from the tubulin−colchicine crystal structure (*21*) obtained from the Protein Data Bank (PDB ID 1SA0).

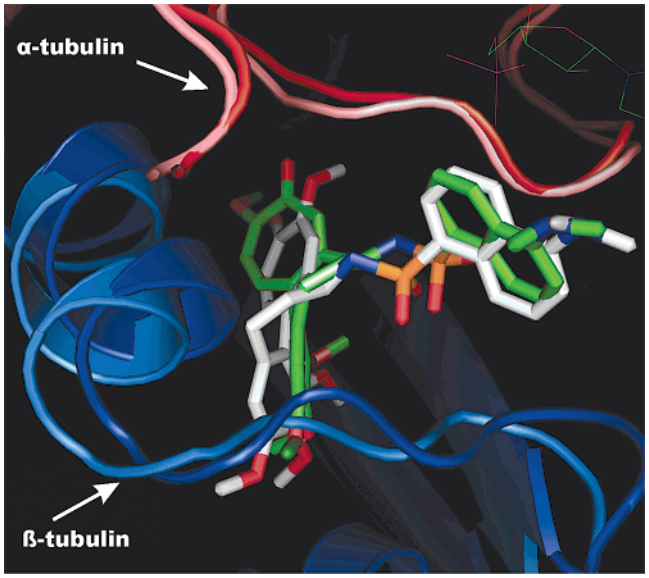

FIGURE 12: Comparison of the models of tubulin complexed with dansyl derivatives of colchicine (white) and isocolchicine (green). The differences in the orientation of the colchicine part of both the drugs can be observed, while the dansyl group assume a nearly identical orientation. The large differences in the conformation of *β* tubulin (represented in shades of blue) are in contrast with negligible changes in the α subunit (represented in shades of red). Darker shades have been used for the -NH-dansyl colchicine complex.

cal properties of the -NH-dansyl colchicine and -NH-dansyl isocolchicine.

Therefore, in conclusion, -NH-dansyl isocolchicine behaves as a bifunctional colchicine analogue, whereby its improved tubulin-binding affinity is attained through its A and B rings, while the C ring remains inactive.

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