NBD-Isocolcemid−Tubulin Interaction: A Novel One-Step Reaction Involving No Conformational Adjustment of Reactants†

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ABSTRACT: Isocolcemid, a colcemid analogue in which the positions of the C-ring methoxy and carbonyl are exchanged, is virtually inactive in binding to tubulin and inhibiting the formation of microtubule assembly. We have found that the substitution of a NBD group in the side chain of the B-ring of isocolcemid can reverse the effect of these structural alterations (at the C-ring) and the newly synthesized NBD-isocolcemid restores the lost biological activity. It inhibits microtubule assembly with an IC_{50} of 12 μM and competes efficiently with [^{3}H]colchicine, for binding to tubulin. NBD-isocolcemid has two binding sites on tubulin; one is characterized by fast binding, whereas the binding to the other site is slow. These two sites are independent and unrelated to each other. Colchicine and its analogues compete with NBD-isocolcemid for the slow site. Association and dissociation rate constants for the fast site, obtained from the stopped-flow measurements, are $(7.37 \pm 0.70) \times 10^5$ M^{-1} s^{-1} and 7.82 ± 2.74 s^{-1}, respectively. While the interaction of colchicine and its analogues with tubulin involves two steps, NBD-isocolcemid binding to tubulin at the slow site has been found to be a one-step reaction. This is evident from the linear dependence of the observed rate constant (k_{obs}) with both NBD-isocolcemid and tubulin concentrations. The interaction of NBD-isocolcemid with tubulin does not involve the conformational change of NBD-isocolcemid, as is evident from the unchanged CD spectra of the drug. The absence of enhanced GTPase activity of tubulin and the native-like protease cleavage pattern of the NBD-isocolcemid−tubulin complex suggest an unaltered conformation of tubulin upon NBD-isocolcemid binding to it as well. Implications of this on the mechanism of polymerization inhibition have been discussed.

Colchicine binds to dimeric tubulin with a stoichiometry of 1:1. This binding of colchicine and its structural analogues disrupts the organized assembly of tubulin dimers into microtubules. Colchicine, being an antimitotic drug, possesses immense therapeutic importance. Thus, the mechanism of colchicine−tubulin interaction has been a topic of intense study for the last several years (*1*, *2*).

The currently accepted mechanism for this process, as proposed by Garland (*1*) and subsequently tested by others, is composed of two sequential binding steps as illustrated below:

$$T + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [TC] \xrightarrow{k_2} [TC]^*$$

The first step involves an initial rapid equilibrium between colchicine (C) and tubulin (T) yielding a nonfluorescent low-affinity complex [TC], while the second step is a slow and poorly reversible process giving rise to the high-affinity fluorescent complex [TC]*.

Isocolchicine, a structural isomer of colchicine, differs from colchicine in the C-ring, where the positions of methoxy and carbonyl groups are reversed. Early studies indicated that isocolchicine and its structural analogues are inactive in binding to tubulin (*3*, *4*) as they are unable to inhibit either the microtubule assembly (*5*) or the binding of [^{3}H]colchicine to tubulin (*6*). However, a detailed study by Hastie et al. (*7*) showed that isocolchicine does bind tubulin at the colchicine site with an affinity that is much lower (500-fold lower) than that of colchicine and that inhibits tubulin polymerization with an IC_{50} of 1 mM. They have suggested that although it forms the low-affinity complex at the preassociation step, the final complex, formed in the second step, is never attained.

Our results in this report show that the introduction of a NBD[1] moiety in the B-ring of the isocolcemid molecule (side chain at the C-7 position) enhances the biological activity of the otherwise poorly active isocolcemid. Thus, NBD-isocolcemid (Figure 1) can inhibit tubulin assembly efficiently and competes with [^{3}H]colchicine for binding to tubulin. The most important observation of this study is that while the interaction of colchicine and all its analogues with tubulin is a two-step process, the interaction of NBD-

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[1] Abbreviations: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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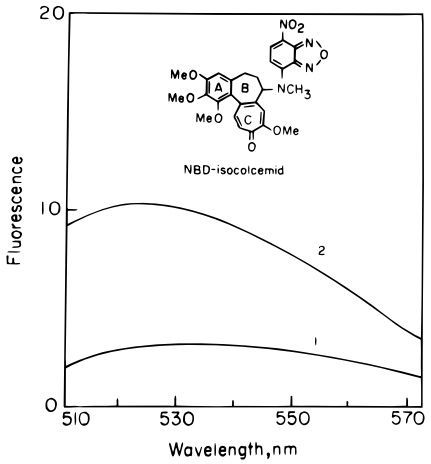

FIGURE 1: Structure of NBD-isocolcemid and its binding with tubulin. Fluorescence emission spectra of (1) 5 μM NBD-isocolcemid and (2) the tubulin−NBD-isocolcemid complex (5 μM each). The complex was prepared in buffer A by incubating the mixture for 10 min at 25 °C. The excitation wavelength was 470 nm.

isocolcemid with tubulin is a one-step process, as is evident from the kinetic data. The second step of the colchicine−tubulin interaction where the conformational adjustments of both the drug and the tubulin take place is absent in the NBD-isocolcemid−tubulin interaction.

MATERIALS AND METHODS

NBD-colcemid, 4-[*N*-(iodoacetoxyethyl)-*N*-methylamino]-NBD, and the sodium salt of 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]ethanesulfonic acid (NBD-taurine) were purchased from Molecular Probes (Eugene, OR). Colchicine, podophyllotoxin, GTP, EGTA, Pipes, and polyester-bound TLC silica gel plates were from Sigma Chemical Co. (St. Louis, MO). Radioactive colchicine (^{3}H-labeled C-ring methoxy, 70.0 Ci/mmol) was obtained from New England Nuclear.

Isocolchicine was synthesized as described by Hastie et al. (*7*). NBD-isocolcemid was a gift from S. Bane Hastie (State University of New York, Binghamton, NY); it was synthesized by the method of Hiratsuka and Kato (*8*) from isocolcemid, and its purity was determined by ^{1}H NMR and TLC analysis.

Tubulin Isolation. Microtubular proteins were isolated from goat brains by two cycles of a temperature-dependent assembly−disassembly process. Pure tubulin was isolated from it using glutamate buffer for assembly (*9*). The composition of the disassembly buffer was 0.05 M Pipes (pH 6.9) containing 0.5 mM $MgCl_2$ and 1 mM EGTA. The protein concentrations were determined by the method of Lowry et al. (*10*).

Tubulin Polymerization Assay. Pure tubulin (1.5 mg/mL) in Pipes buffer was polymerized at 37 °C in the presence of 1 mM GTP. Polymerization was initiated using 8% dimethyl sulfoxide (Me_2SO), and the turbidity was measured by absorbance at 390 nm rather than at the usual 350 nm, as both NBD-colcemid and NBD-isocolcemid have absorption maxima near 350 nm. A Shimadzu UV-160 double-beam spectrophotometer, fitted with a temperature-controlled circulating water bath, was used for this purpose. IC_{50} was calculated as the concentration of drug causing 50% inhibition of polymer mass.

Fluorescence Studies. Fluorescence spectra were recorded in a Hitachi F-3000 fluorescence spectrophotometer connected to a constant-temperature circulating water bath. When fluorescence enhancement of drug was monitored, the excitation and emission wavelengths were 465 and 530 nm, respectively, for both NBD-colcemid and NBD-isocolcemid. Excitation and emission band-pass were 3 and 20 nm, respectively.

Corrections for the inner filter effect were carried out using the following equation

$$F_{corr} = F_{obs}\{\text{antilog}[(A_{ex} + A_{em})/2]\}$$

where A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance at the emission wavelength.

For the determination of k_{obs}, the unknown parameters were varied and the best fit values giving minimum χ^2 values were obtained by the method of iteration, using a basic program written for this purpose.

For the plot depicting the variation of k_{obs} with reactant concentration, each point represents the mean of five experiments and the best fit was obtained using ENZFITTER, a nonlinear regression data analysis program from BIOSOFT.

Circular Dichroism. Circular dichroism studies were carried out on a Jasco J600 spectropolarimeter. Each spectrum was recorded as an average of six scans. For measurement of CD spectra of the drug−tubulin complex, the free drugs were separated from the complex by rapid gel filtration using Sephadex G-25.

[^{3}H]Colchicine Binding Assay. The [^{3}H]colchicine binding assays were performed by the method of Banerjee and Bhattacharyya (*11*).

Fast-Reaction Kinetic Studies. The fast phase of the kinetics of association of NBD-isocolcemid with tubulin was studied using a SX18.MV kinetic spectrometer from Applied Photophysics Ltd. Samples were excited at 445 nm with the help of a cutoff filter, and the emission spectra were recorded at 530 nm. The dead time of the instrument was determined to be 2 ms. Experiments were performed under pseudo-first-order kinetic conditions with 2.5 μM NBD-isocolcemid and 20−60 μM tubulin (final concentration after mixing). The kinetic curves were analyzed using nonlinear least-squares fitting.

GTPase Assay. The effect of various colchicine analogues on tubulin-mediated hydrolysis of GTP was assessed essentially as described by Monasterio and Timasheff (*12*). Tubulin samples (10 μM) were prepared in Pipes buffer with 100 μM colchicine analogues. Samples were preincubated at 25 °C for 20 min. The [^{32}P]GTP (0.1 mM) was added to each sample and the mixture incubated at 37 °C. Aliquots (50 μL) were withdrawn at different time intervals, and the reaction was quenched with 50 μL of 20% perchloric acid (PCA). Samples were centrifuged, and the supernatant was added to a mixture of 0.6 mM KH_2PO_4 (1 mL) and 5% ammonium molybdate in 2 M H_2SO_4 (0.5 mL). The samples were extracted with 2 mL of cyclohexane and an isobutanol mixture (1:1), and 100 μL from the organic layer was counted for radioactivity.

Limited Protease Digestion. The limited protease digestion was carried out according to the method of Sackett and Varma (*13*). Briefly, tubulin (10 μM) was incubated without or with colchicine, NBD-colcemid, and NBD-isocolcemid

separately for 30 min at 30 °C. Drug concentrations were 0.1 mM in the reaction mixture. Samples were digested for 3 min only at 30 °C with chymotrypsin (0.01 mg/mL), keeping the tubulin to enzyme ratio at 100:1. The reactions were stopped by adding 0.2 mM PMSF. Samples were analyzed by SDS−PAGE (12% polyacrylamide gel) and followed by Coomassie staining.

Determination of Binding Parameters. The dissociation constant and stoichiometry of NBD-isocolcemid binding to the tubulin−colchicine complex (TC) were determined from a conventional Scatchard plot (*14*). Fluorescence enhancement (Q) was calculated from the double-reciprocal plot (*15*) using the equation

$$F_o/(F - F_o) = [K_d/(Q - 1)](1/TC_T) + 1/(Q - 1)$$

where F_o is the fluorescence of a fixed concentration of NBD-isocolcemid, F is its fluorescence at a particular concentration of the tubulin−colchicine complex (TC), and TC_T is the total concentration of the tubulin−colchicine complex. The TC used in reverse titration was always in excess over the NBD-isocolcemid concentration, and an approximation was made that the total concentration of TC is almost equal to the free TC concentration. Q at 530 nm was calculated by titrating a fixed amount (0.5 μM) of NBD-isocolcemid with increasing amounts of TC (1−10 μM). The stoichiometry and K_d of NBD-isocolcemid binding to TC were determined from a Scatchard plot using 2 μM TC and varying the NBD-isocolcemid concentration over the range of 0.2−20 μM at 37 °C.

The TC-bound NBD-isocolcemid concentrations were calculated by the method of Mas and Colman (*16*) using the equation

$$[TCD] = [D_T/(Q - 1)][(F/F_o) - 1]$$

where [TCD] is the TC-bound NBD-isocolcemid concentration, D_T is the total NBD-isocolcemid concentration, F is the fluorescence value at a fixed concentration of TC and a particular concentration of NBD-isocolcemid, and F_o is the fluorescence of the same concentration of NBD-isocolcemid without the TC. Q determined from the double-reciprocal plot was used. The value of TC-bound NBD-isocolcemid concentration thus obtained was used for Scatchard analysis. In these experiments, an excitation wavelength of 500 nm was used to avoid the inner filter effect.

Solution Conditions. All experiments were carried out in either Pipes or 10 mM phosphate (pH 6.9) containing 0.25 mM $MgCl_2$ and 0.5 mM EGTA (buffer A), unless stated otherwise. Stock solutions of NBD-colcemid and NBD-isocolcemid were prepared in absolute ethanol and stored at −20 °C. Dilute solutions, if required, were prepared in buffer A immediately before use. As the NBD derivatives are prone to photodecomposition, their purities were checked by TLC.

RESULTS

Binding of NBD-Isocolcemid to Tubulin. Binding of NBD-isocolcemid (see Figure 1 for the structure) to tubulin was studied using fluorescence of the NBD group. Binding to tubulin enhanced the NBD-isocolcemid fluorescence intensity with a large blue shift of the emission maximum. The emission maximum of free NBD-isocolcemid shifted 9 nm upon binding to tubulin (λ_{max} went from 531 to 522 nm). The enhancement of fluorescence intensity and the shift of λ_{max} occur with the time of incubation of NBD-isocolcemid and tubulin at 25 °C (Figure 1). The observed binding of NBD-isocolcemid to tubulin is not solely due to the presence of the NBD group, as NBD compounds lacking the colchicine or colcemid moiety such as the 2-mercaptoethanol conjugate of 4-[*N*-(iodoacetoxyethyl)-*N*-methylamino]-NBD and NBD-taurine did not bind to tubulin (Table 1). There were no changes in fluorescence intensity or emission maximum when tubulin and any one of these compounds were incubated together at 25 °C. These results indicate that the binding (and consequent effect of enhanced fluorescence and the shift of λ_{max}) of NBD-isocolcemid to tubulin is a combined effect of both the NBD group and the isocolchicine nucleus. The time dependence of NBD-isocolcemid binding was tested by observing its fluorescence as a function of time after mixing 3 μM tubulin with 5 μM NBD-isocolcemid at 37 °C. The profile of fluorescence increase is (Figure 2A) similar to that for the NBD-colcemid−tubulin interaction reported from this laboratory (*17*). It is characterized by a very rapid phase which is completed within the time of mixing of the protein and the drug, and a slow phase like

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

NBD derivative	fluorescence without tubulin	fluorescence with tubulin
4-[*N*-(iodoacetoxyethyl)-*N*-methylamino]-NBD-2-mercaptoethanol	14	16
NBD-taurine	71	75
NBD-colcimid	28	80
NBD-isocolcimid	27	80

[a] Tubulin (5 μM) was mixed with different NBD derivatives (5 μM) in PEM buffer at 25 °C. Fluorescence was measured after incubation for 10 min at 25 °C. The excitation wavelength was 470 nm.

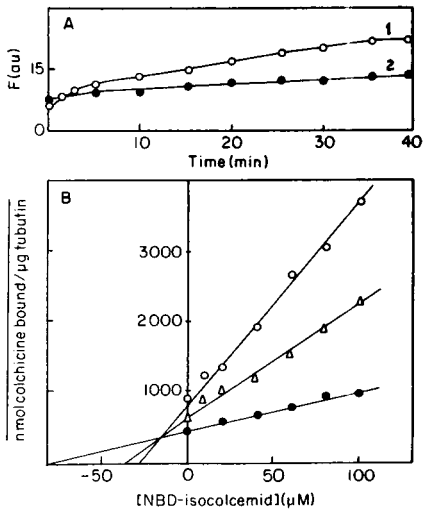

FIGURE 2: Time-dependent binding of NBD-isocolcemid to tubulin and the modified Dixon plot. (A) The tubulin−colchicine complex was prepared by incubating 3 μM tubulin and 30 μM colchicine at 37 °C for 90 min. Binding of NBD-isocolcemid to tubulin (○) and the tubulin−colchicine (●) complex was followed for 40 min at 37 °C. Excitation and emission wavelengths were 465 and 530 nm, respectively. (B) Modified Dixon plot. The concentrations of [^{3}H]-colchicine were 1.0 (○), 2.0 (△), and 5.0 μM (●). The reaction mixture contained tubulin (2 μM) and NBD-isocolcemid, at the indicated concentration, and they were incubated at 25 °C for 90 min.

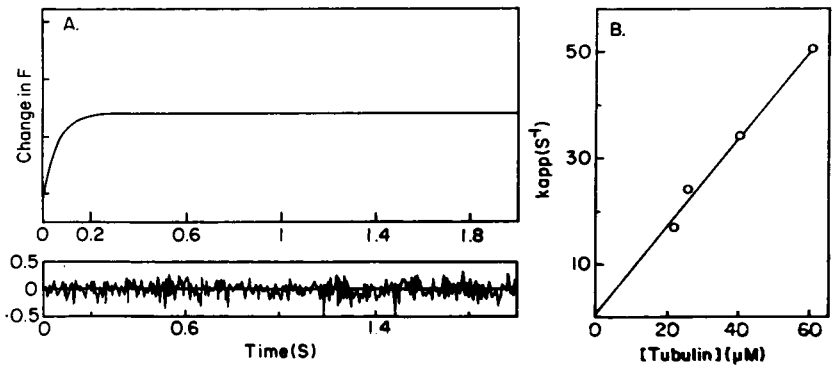

FIGURE 3: NBD-isocolcemid binding to tubulin using stopped-flow analysis. (A) Best fit line to the fluorescence increase (actual fluorescence points not shown) for the binding of NBD-isocolcemid and tubulin at 25 °C. NBD-isocolcemid (5 μM) and tubulin (40 μM) were mixed, and the fluorescence change was recorded. The samples were excited at 465 nm. The residuals between the experimental and theoretical curves are shown below. (B) Determination of the rate constant for the association of NBD-isocolcemid with tubulin at 25 °C. K_{app} values were plotted as a function of tubulin concentration after mixing. The resultant concentration of NBD-isocolcemid was 2.5 μM.

the type which is observed for the colchicine binding to tubulin. NBD-isocolcemid also binds to tubulin whose colchicine binding site is occupied by colchicine (curve 2 of Figure 2A) or its analogues such as podophyllotoxin and nocodazole (data not shown). In all three cases, while the fast phase of binding remains unaffected, the magnitude of the slow time-dependent phase of the fluorescence increase has been lowered significantly. These results indicate that like NBD-colcemid, there are two NBD-isocolcemid binding sites on tubulin and that colchicine, podophyllotoxin, and nocodazole compete for the site responsible for the slower phase of the NBD-isocolcemid−tubulin interaction. The stoichiometry obtained from the Scatchard plot of NBD-isocolcemid binding to the preformed tubulin−colchicine complex was 0.66 (data not shown). The affinity constant obtained from the Scatchard plot is 1.1×10^5 M^{-1} for NBD-isocolcemid binding to the fast site. It is to be noted that while NBD-isocolcemid can bind to both sites (fast and slow) as well as colchicine-bound tubulin, colchicine cannot bind NBD-colcemid-bound tubulin. Thus, the pretreatment of tubulin with NBD-isocolcemid abolished the [^{3}H]colchicine binding of the protein (data not shown). These experiments, however, do not rule out the possibility of binding of colchicine and its analogues to the fast site. It is possible that they bind to the fast site reversibly and with a lower affinity.

Competitive Inhibition of [^{3}H]Colchicine Binding to Tubulin by NBD-Isocolcemid. To test whether the slower site (Figure 2A,B) is due to the drug binding to the colchicine site, NBD-isocolcemid was allowed to compete with [^{3}H]-colchicine 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 [^{3}H]colchicine binding is inhibited competitively by NBD-isocolcemid, yielding an apparent K_i value of 15 μM. The highest concentration of NBD-isocolcemid used in this experiment was 100 μM, and under similar conditions, even 100 μM isocolchicine hardly caused any detectable inhibition of [^{3}H]-colchicine binding (data not shown).

Stopped-Flow Study of the Kinetics for the Fast Site. A representative stopped-flow fluorescence trace for the association of NBD-isocolcemid with tubulin, for the fast site, is shown in Figure 3A. The approach of the fluorescence to its final value was essentially a monophasic first-order process. The rate constant for the first-order approximation (k_{app}) was obtained by fitting the data to a single-exponential function as 34.8 s^{-1}.

The kinetic data under pseudo-first-order conditions were interpreted in terms of a single-step reaction.

$$T + C \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} TC \qquad (1)$$

where T and C refer to tubulin and NBD-isocolcemid and k_1 and k_{-1} are the association and dissociation rate constants, respectively. Equation 2 relates the observed rate constant (K_{app}) to k_1 and k_{-1} and the component in excess, i.e., [T].

$$k_{app} = k_1[T] + k_{-1} \qquad (2)$$

The values of k_1 and k_{-1} were determined from the slope and intercept, respectively, of the linear plots of k_{app} versus [T] (Figure 3B). The values of k_1 and k_{-1} thus obtained are $(7.37 \pm 0.70) \times 10^5$ M^{-1} s^{-1} and 7.82 ± 2.74 s^{-1}, respectively. These values are similar to that obtained for the tubulin−NBD-colcemid interaction (*17*). The value of the dissociation constant could not be directly determined due to the lack of the displacing ligand, as neither colchicine nor its derivatives were able to displace NBD-isocolcemid bound to tubulin at the fast site. However, the dissociation constant (K_d) determined from the ratio of two rate constants is $(10.6 \pm 2.6) \times 10^{-6}$ M.

Kinetics for the Slow Site and Dependence of k_{obs} on Reactant Concentration. It is well established that colchicine and its analogues bind tubulin in a two-step mechanism which involves an initial fast step with the formation of the low-affinity complex (TC), followed by a slow drug-induced conformational change in tubulin, resulting in the formation of the stable fluorescent complex (TC)* (*1*, *2*)

$$T + C \overset{K_1}{\Leftrightarrow} TC \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} (TC)^* \qquad (3)$$

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

The rate constants for the two-step scheme presented above under pseudo-first-order conditions are given by the following hyperbolic expression (*1*).

$$k_{obs} = \frac{K_1 k_2 [C]}{1 + K_1 [C]} + k_{-2} \quad (4)$$

Under pseudo-first-order conditions, the k_{obs} exhibits a nonlinear dependence on both drug and protein concentration (*1*). The two-step mechanism of the colchicine−tubulin interaction is based on this observation. When the dependence of k_{obs} on reactant concentration for the NBD-isocolcemid−tubulin interaction was determined, we found that it is a linear function of both ligand and protein concentration (Figure 4A). Interestingly, for the tubulin−NBD-colcemid interaction, the k_{obs} is a nonlinear function of ligand concentration (Figure 4B), similar to that for the colchicine−tubulin interaction. These results suggest that while the NBD-colcemid−tubulin interaction is a two-step process, the NBD-isocolcemid−tubulin interaction is a single-step reaction. Thus, the reaction of these two drugs can be represented as

$$\text{tubulin} + \text{NBD-colcemid} \xrightarrow[\text{(preassociation)}]{\text{step 1}} [\text{tubulin}-\text{NBD-colcemid}]$$

$$[\text{tubulin}-\text{NBD-colcemid}] \xrightarrow[\text{(conformational change)}]{\text{step 2}} \text{tubulin}-\text{NBD-colcemid}$$

$$\text{tubulin} + \text{NBD-isocolcemid} \xrightarrow{\text{(single step)}} \text{tubulin}-\text{NBD-isocolcemid}$$

All colchicine analogues studied so far have exhibited a two-step binding reaction with tubulin (*18*−*21*). However, a one-step binding mechanism has been suggested for the isocolchicine−tubulin interaction (*7*). This suggestion was based upon the structure analyses of isocolchicine conformers. Our conclusion regarding the one-step binding mechanism for NBD-isocolcemid is however based on kinetic data.

Conformational Changes of Tubulin and NBD-Isocolcemid upon Their Interaction. Colchicine binding to tubulin was found to consist of two steps: a rapid equilibration of the protein and the drug which forms a low-affinity complex, which is followed by a slow, essentially irreversible process which forms the fluorescent complex. The conversion of the low-affinity complex to the fluorescent complex involved conformational changes of tubulin as well as colchicine, and substantial data now support this proposal (*1*, *3*, *13*, *22*−*26*). We have tested whether both tubulin and NBD-isocolcemid undergo conformational adjustments when they interact with one another. The CD spectra of tubulin with NBD-colcemid and NBD-isocolcemid are shown in Figure 5. NBD-isocolcemid has negative CD bands due to the isocolcemid moiety at 330 nm and for the NBD group at 479 nm. Binding of tubulin does not effect any one of the bands significantly (Figure 5A). NBD-colcemid, which also possesses two CD bands for the colcemid and NBD nucleus, however, exhibited reductions in both negative CD bands when reacted with tubulin (Figure 5B). We have tested the changes on tubulin conformations upon NBD-isocolcemid binding to it. Chymotrypsin cleaves β-tubulin only at Tyr 281, producing fragments βN and βC (lane 2, Figure 6). However, it is shown by Sackett and Varma (*13*) that the colchicine-bound tubulin has an additional chymotrypsin sensitive site at or near the C-terminus of tubulin. No such additional site was detected when isocolchicine-treated tubulin was used (*13*). We have attempted to find out whether such an additional chymotrypsin sensitive site is generated upon NBD-isocolcemid binding. Chymotrypsin has been chosen for this purpose as colchicine and its analogues are known to bind the β-subunit of tubulin. Enzymatic digestion was carried out for 3 min at 30 °C with a chymotrypsin to tubulin ratio of 1:100. Figure 6 shows the SDS−PAGE analysis of chymotrypsin digest of colchicine−tubulin (lane 3), NBD-colcemid−tubulin (lane 4), and NBD-isocolcemid−tubulin complexes (lane 5). As is evident from the photograph, both colchicine and NBD-colcemid complexes exhibit an additional band (indicated by the arrow), whereas unliganded tubulin and the NBD-isocolcemid−tubulin complex exhibit no band in that position. This suggests that NBD-isocolcemid binding to tubulin is unable to induce conformational changes similar to those induced by colchicine. It is known that colchicine and other colchicine site ligands stimulate tubulin GTPase activity. This enhanced enzymatic activity of liganded tubulin is a consequence of the drug-induced conformational changes on tubulin (*27*−*29*). What happens to the GTPase activity if NBD-isocolcemid or NBD-colcemid is bound to tubulin? This is addressed in Figure 7. The hydrolysis of GTP by tubulin is enhanced by colchicine (curve b) and NBD-colcemid (curve c). However, NBD-isocolcemid (curve e) and isocolchicine (curve d) binding have no effect on GTPase activity of tubulin (curve a shows the GTPase activity of free tubulin).

The tubulin dimer has 20 sulfhydryl groups, and all of them react with DTNB, although with different rates (unpublished results). Due to the presence of a large number of sulfhydryl groups, it is difficult to analyze the composite rate profile in terms of the individual sulfhydryl groups. The reaction reaches a plateau by about 30 min. Colchicine has a profound influence on the rate and extent of DTNB reaction. It appears that at least two sulfhydryl groups are completely protected in the presence of colchicine. In addition, the rate profile indicates that several others react at a slower rate. It has been shown that only one sulfhydryl group is essential for colchicine binding. Thus, one may conclude that colchicine-induced conformational change is responsible for the slower reactivity of some of the sulfhydryl groups. The rates of reaction of tubulin−NBD-colcemid and tubulin−NBD-isocolcemid complexes were measured; it was observed that the rate profile of DTNB reaction is similar to that of the colchicine complex for NBD-colcemid, whereas the rate profile is similar to that of the native tubulin for NBD-isocolcemid (data not shown). This indicates that NBD-isocolcemid is unable to induce the same kind of conformational change in the protein that colchicine and NBD-colcemid do.

Inhibition of Tubulin Polymerization by NBD-Isocolcemid. As isocolchicine was reported to inhibit tubulin self-assembly in the millimolar range, we have tested NBD-isocolcemid toward inhibition of tubulin polymerization using dimethyl sulfoxide (Me_2SO). NBD-isocolcemid shows (Figure 8) progressive concentration-dependent inhibition of tubulin self-assembly with an IC_{50} of 12 μM, and 75% inhibition was obtained with 20 μM NBD-isocolcemid (inset of Figure 8). Under similar conditions, even 100 μM isocolchicine does not inhibit tubulin polymerization significantly (data not shown). The reported IC_{50} for the isocolchicine inhibition

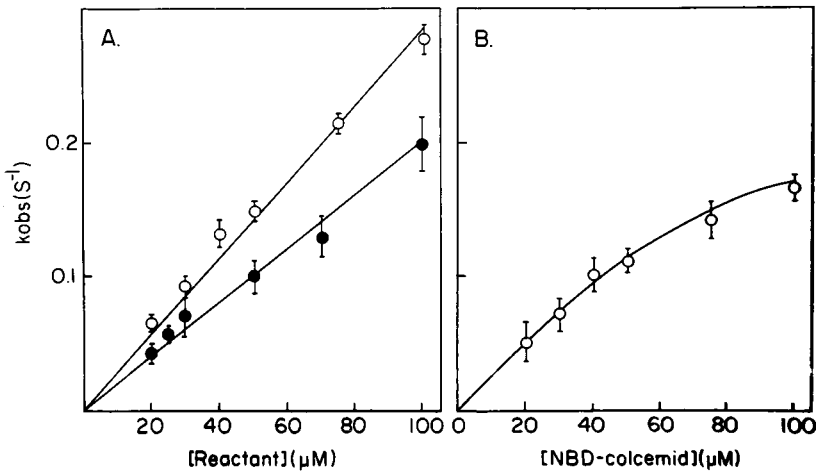

FIGURE 4: Dependence of the observed rate constant (k_{obs}) on the concentrations of the reactants. (A) k_{obs} for the tubulin−NBD-isocolcemid reaction was plotted against tubulin (●) and NBD-isocolcemid (○) concentrations. (B) k_{obs} for the tubulin−NBD-colcemid reaction was plotted against the NBD-colcemid concentration (○). The tubulin concentration was 1 μM, in the case when drug concentrations were varied. The NBD-isocolcemid concentration was 2 μM when tubulin concentrations were varied. Excitation and emission wavelengths were 465 and 530 nm, respectively.

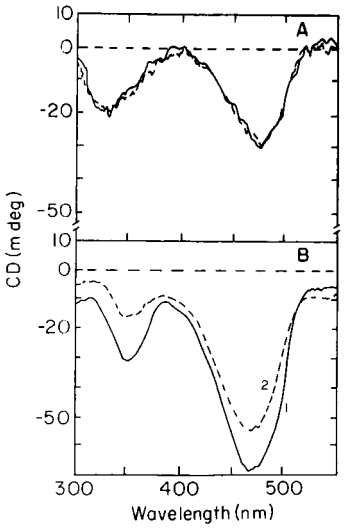

FIGURE 5: Circular dichroism spectra of NBD-isocolcemid and NBD-colcemid, in the presence or absence of tubulin. (A) Spectra of 10 μM NBD-isocolcemid (—) and 10 μM tubulin−isocolcemid complex (- - -) are shown. (B) Spectra of 10 μM NBD-colcemid (1) (—) and 10 μM tubulin−NBD-colcemid complex (2) (- - -) are shown. The drug−protein complexes were prepared by mixing 10 μM drug with 10 μM tubulin, and incubating for 60 min at 25 °C. Free drugs were separated from the protein−drug complex by gel filtration.

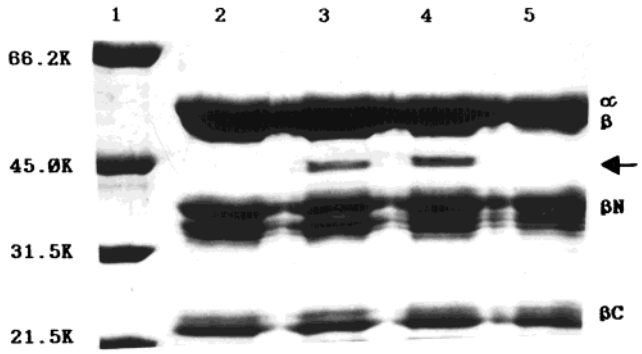

FIGURE 6: Limited proteolysis of drug−tubulin complexes by chymotrypsin: lane 1, molecular weight markers [serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), and trypsin inhibitor (21 500)]; lane 2, tubulin without drug (control); lane 3, tubulin−colchicine complex; lane 4, tubulin−NBD-colcemid complex; and lane 5, tubulin−NBD-isocolcemid complex. Sample preparations, digestion conditions, and SDS−PAGE conditions are described in Materials and Methods.

of tubulin polymerization into microtubules is 1000 μM (*7*).

DISCUSSION

Isocolcemid is a structural isomer of colcemid, differing in the relative positions of the C-ring methoxy and carbonyl groups. On the basis of several studies, analogues belonging to the isoseries of both colchicine and colcemid were believed to be inactive in binding to tubulin and inhibiting microtubule assembly. In the binding study with tubulin presented here, we have used an isocolcemid analogue which contains a NBD group at the C-7 position (B-ring side chain) of the molecule. Remarkable observations of this binding study are as follows. (i) NBD-isocolcemid inhibits tubulin polymerization efficiently with an IC_{50} value of 12 μM (Figure 8). (ii) NBD-isocolcemid competes with [^{3}H]colchicine for binding to tubulin with a K_i of 15 μM (Figure 2B). (iii) Unlike colchicine and colcemid, NBD-isocolcemid has two sites of binding (one fast and the other slow) to tubulin. Colchicine and its analogues compete with the NBD-isocolcemid for the slow site (Figure 2A). (iv) While the binding of colchicine and its analogues with tubulin involves the conformational changes of tubulin (i.e., enhanced GTPase activity of tubulin and the generation of an additional chymotrypsin site when colchicine binds to it) and the drug molecule (CD of free colchicine is abolished when it is bound to tubulin), no such conformational adjustments of tubulin and NBD-isocolcemid were detected (Figures 5−7). These results are substantiated by kinetic data where we observed the linear dependence of k_{obs} with increasing concentrations of NBD-isocolcemid or tubulin concentration (Figure 4A).

A detailed study by Hastie et al. (*7*) showed that isocolchicine inhibits tubulin self-assembly into microtubules with an IC_{50} of about 1000 μM, and competes poorly with [^{3}H]colchicine for binding to tubulin. The affinity of isocolchicine for the colchicine receptor site is 5.5×10^3 M^{-1}, which is 500-fold lower than that of colchicine (*7*). Binding occurs in one step in a reversible manner and does not

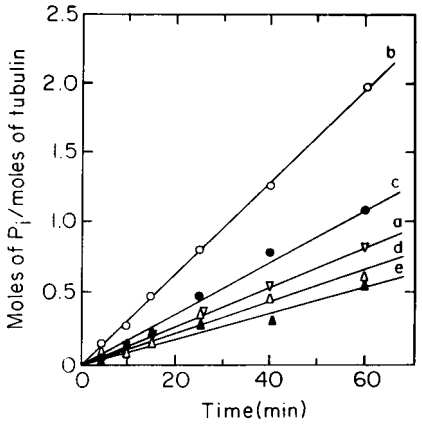

FIGURE 7: Effect of different drugs on GTPase activity of tubulin. GTP hydrolysis by tubulin was studied in the absence (a) and presence of (b) colchicine, (c) NBD-colcemid, (d) isocolchicine, and (e) NBD-isocolcemid. Tubulin (10 μM) was preincubated without or with each ligand (100 μM) separately for 20 min at 25 °C. Then 0.1 mM [^{32}P]GTP was added, and incubation was continued. At various time intervals, 50 μL aliquots were removed and GTP hydrolysis was assayed as described in Materials and Methods.

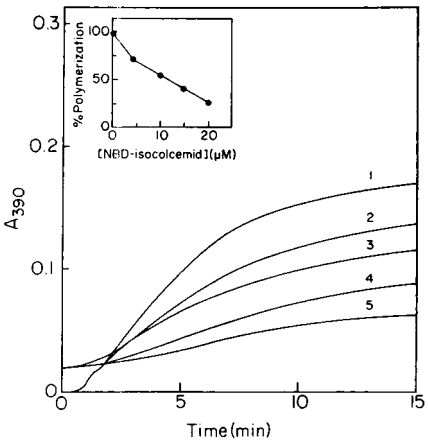

FIGURE 8: Effect of NBD-isocolcemid on tubulin polymerization. Tubulin (15 μM) in buffer A containing 1 mM GTP at 37 °C was mixed with NBD-isocolcemid at different concentrations as follows: (1) 0, (2) 5, (3) 10, (4) 15, and (5) 20 μM. Polymerization was initiated by the addition of 8% dimethyl sulfoxide (Me_2SO). The inset shows a plot of percent polymerization vs the concentration of NBD-isocolcemid.

undergo the second slow step (*7*). This second step is responsible for many changes that take place in the drug and protein when they interact (*22*). It was thus anticipated that the poor inhibitory property of isocolchicine was due to its inability to undergo the changes that take place in the second step of binding. NBD-isocolcemid is thus an interesting colchicinoid which throws light in this aspect. Like that of isocolchicine, the first step of NBD-isocolcemid binding to tubulin is not followed by the step involving the conformational adjustments. The inhibitory property of NBD-isocolcemid clearly suggests that such conformational changes are probably not related to the microtubule inhibition, but it is the affinity of the drug−tubulin interaction that determines the potency of inhibition. This is supported by the fact that isocolchicine which does not undergo the second slow step is also an inhibitor, albeit a poor one (*7*). Recently, two low-affinity isocolchicine binding sites for binding to tubulin have been reported (*30*). The mechanism by which these two sites are related to the two binding sites of NBD-isocolcemid reported here requires further study with several isocolchicine analogues.

Structure−activity studies indicate that the A- and C-rings of colchicine comprise the minimum structural features of the molecule necessary for high-affinity binding to tubulin. On the basis of several model compound studies, it has been established that the B-ring of colchicine is not essential for binding to tubulin; nevertheless, the presence of the B-ring and its side chain play an important role in the kinetics and energetics of the reaction (*19*, *21*, *31*−*34*). In this context, the results presented here are unique in that biological activity, induced by alterations in the C-ring, can be restored by a hydrophobic moiety (NBD group) substitution in the B-ring, the "nonessential" part of the molecule. On the basis of the binding of tubulin with a large number of B-ring analogues of colchicine, it was hypothesised by Bhattacharyya et al. (*33*) that the acetamido group at the C-7 position may be a determinant factor recognized by the colchicine binding site. The NBD group has considerable hydrophobic character and may provide excess binding energy, through hydrophobic interactions, thus strengthening the binding. The NBD group may also have other entropic advantages (like chelate effect) toward binding over the isocolcemid nucleus alone. The mechanism by which the substitution of the NBD group in the nonessential part (ring-B) of a molecule enhance the affinity needs further study with several B-ring analogues of isocolchicine or isocolcemid.

In conclusion, we report here the first isocolcemid analogue that competes with [^{3}H]colchicine for binding to tubulin and inhibits tubulin polymerization at low concentrations, suggesting a new direction in the structure−function relationship of colchicine analogues.

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