The Binding of Isocolchicine to Tubulin

MECHANISMS OF LIGAND ASSOCIATION WITH TUBULIN*

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Isocolchicine is a structurally related isomer of colchicine altered in the methoxytropone C ring. In spite of virtual structural homology of colchicine and isocolchicine, isocolchicine is commonly believed to be inactive in binding to tubulin and inhibiting microtubule assembly. We have found that isocolchicine does indeed bind to the colchicine site on tubulin, as demonstrated by its ability to competitively inhibit \[^{3}H\]colchicine binding to tubulin with a \(K_d\) \(\approx 400 \mu M\). Isocolchicine inhibits tubulin assembly into microtubules with an \(I_50\) of about 1 mM, but the affinity of isocolchicine for the colchicine receptor site, \(5.5 \pm 0.9 \times 10^3 \text{ m}^{-1}\) at \(23^\circ\text{C}\), is much less (\(-500\)-fold) than that of colchicine. Unlike colchicine, isocolchicine binds rapidly, and the absorption and fluorescence properties of the complex are only modestly altered compared to free ligand. It is proposed that the binding of isocolchicine to tubulin may be rationalized either in terms of conformational states of colchicinoids when liganded to tubulin or by the structural requirements for C-10 substituents for high affinity binding to the colchicine receptor.

Tubulin is the target protein for a large number of drugs, which possess a wide range of therapeutic utilities. Binding to tubulin, a 100,000-Da protein consisting of two similar (α and β) subunits, typically disrupts organized assembly of tubulin monomers into microtubules. The precise mechanism(s) by which these drugs, which include colchicine, podophyllotoxin, nocodazole, and related benzimidazole systems, the vinca alkaloids, maytansinoids, and taxol, disturb normal tubulin polymerization is unknown and the topic of intense study (1–3). The first agent demonstrated to bind to tubulin was colchicine 1, which led in turn to the identification of tubule-associated proteins; PM buffer, 100 mM Pipes, 1.0 mM MgSO\(_4\), 0.1 mM GTP, 2.0 mM dithioerythritol (4).

The colchicine-tubulin interaction is characterized by several unusual features, including the strong induction of colchicine tropone fluorescence (5) and the loss of colchicine tropone (UV-CD) chirality (6) upon binding and the slow formation of a poorly reversible complex (7, 8). The kinetics of the interaction has been suggested to be modeled by the two-step sequence of binding illustrated below (9, 10):

\[
K_1 \quad T + C \rightleftharpoons [T.C] \quad k_2 \quad [T.C*]
\]

(1)

This sequence was postulated to involve an initial rapid equilibrium between colchicine [C] and tubulin [T] to form a low affinity colchicine-tubulin complex [T·C], which proceeds to the fluorescent species [T·C*] in a slow, essentially irreversible process (9). The conversion of [T·C] to [T·C*] was proposed to involve a conformational change of the protein (9), and substantial data now support this proposal (7, 11–15).

Isocolchicine 2, a semisynthetic structural isomer of colchicine differing in the relative positions of the C-ring methoxy and carbonyl moieties, has virtual structural homology with the parent by both x-ray (16) and molecular modeling analysis (see Fig. 1 for structures). However, compared to the natural series, isocolchicine and its analogs were reported to be essentially inactive in inhibiting microtubule polymerization (17) and have been shown to exhibit only a slight inhibition of the binding of \[^{3}H\]colchicine to tubulin (18). These observations have led to a general belief that isocolchicine does not bind to tubulin (19, 20). This envisioned lack of interaction of tubulin with isocolchicine has been particularly puzzling due to the wide range of structural systems capable of binding to this tubulin site. In order to further delineate the structural features required for ligand binding to the colchicine receptor site, we have undertaken a detailed study of the association of isocolchicine with tubulin. Our results demonstrate that isocolchicine does indeed bind to tubulin at the colchicine site, but the association parameters and spectroscopic features of the complex are quite different from those of colchicine. These results are interpreted in terms of possible mechanisms for ligands associating with the colchicine receptor site on tubulin.

EXPERIMENTAL PROCEDURES

Materials—Pipes, EGTA, dithioerythritol, and GTP (type II-S) were obtained from Sigma. Phosphocellulose (Whatman P-11) was previously precrystallized according to the manufacturer’s instructions. \[^{3}H\]Colchicine (37.2 Ci/mmol) was purchased from Du Pont-New England Nuclear. All experiments were performed in PMG buffer (0.1 M Pipes, 2.0 mM EGTA, 1.0 mM MgSO\(_4\), 0.1 mM GTP, 2.0 mM dithioerythritol, pH 6.9).

1 T. L. Macdonald, S. B. Hastie, and W. G. Humphreys, Jr., unpublished data.

2 The abbreviations used are: Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, ethylenbis(oxyethylenetricarbamyl) tetraacetate acid; HPLC, high performance liquid chromatography; MAPs, microtubule-associated proteins; PM buffer, 100 mM Pipes, 1.0 mM MgSO\(_4\), 2.0 mM EGTA, pH 6.9; PMG buffer, 100 mM Pipes, 1.0 mM MgSO\(_4\), 2.0 mM EGTA, 0.1 mM EGTA, 2.0 mM dithioerythritol, pH 6.9.

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thereby minimize the inner filter effect (25).

**Tubulin Purification and Protein Determination**—Tubulin, free of microtubule-associated proteins, was prepared from bovine brain by three cycles of assembly/dissassembly followed by chromatography on phosphocellulose as described previously (21) and stored in aliquots in liquid nitrogen. Prior to use, the frozen protein was rapidly thawed, centrifuged at 5000 × g for 10 min to remove small amounts of denatured protein and then chromatographed on a Sephadex G-25 column (0.9 × 25 cm) equilibrated with PMG buffer or alternate appropriate buffer as indicated. Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278.6 nm of 1.20 (mg/ml)⁻¹·cm⁻¹ (14) or by the method of Bradford (22) calibrated with tubulin as a standard (14). MAPs were isolated as described by Aamodt and Williams (23). Due to the known lability of the colchicine binding site (8), all experiments were performed within 6 h after the protein was initially thawed.

**Synthesis of Isocolchicine**—Isocolchicine was prepared in a single semisynthesis from colchicine as described by Chapman et al. (24).

Isocolchicine and colchicine were separated by preparative thin layer chromatography on silica gel (E. Merck Silica Gel 60, F-254 precoated TLC plates; 2 mm coating thickness) developed with 87:13 (v/v) methylene chloride/methanol. Extreme care was taken to ensure that isocolchicine employed in all experiments was uncontaminated with residual colchicine. Isocolchicine purity was assessed by analytical TLC (E. Merck Silica Gel 60, F-254 precoated, 0.25 mm thickness plates; methylene chloride: methanol 90:10 v/v), HPLC with UV detection at 248 nm (Whatman Partisil 5 ODS-3, 250 × 6.35 mm column; methanol/water/acetonitrile (5:80:15)), and high field NMR spectroscopic analysis (Nicolet 360). Through control analyses, it was demonstrated that the TLC- and NMR-based methods could determine <0.5% colchicine impurity, and the HPLC-based method could determine <0.1% colchicine impurity. The structure of isocolchicine was confirmed by ¹H and ¹³C NMR, mass, and infrared spectroscopic analysis. The absorption spectrum displayed a broad maximum in the near-UV region at 348 nm (ε = 1.627 × 10⁴ M⁻¹·cm⁻¹) in water and in PMG buffer.

**Absorption Spectroscopy**—The absorption spectra of tubulin and isocolchicine were determined with a Cary 218 or a Varian DMS-90 spectrophotometer. For difference spectra, tandem cells (0.437 path length in each compartment) were used in the Cary 218 in the following manner: the front compartments of each cell were filled with degassed PMG buffer containing the chosen concentration of isocolchicine, and the rear compartments with degassed PMG buffer containing the chosen concentration of unbound ligand. The peak pressure for consistent, reproducible pump operation and with a Biogel P-6 column (0.9 × 13 cm) which was equilibrated with filtered, degassed PMG buffer containing the chosen concentration of isocolchicine. This system is a modification of that reported by Williams et al. (27) who employed a Chromegapore MSE-100 column; we were unable to obtain reproducible results with the Chromegapore column, apparently due to substantial protein denaturation during the course of the experiment. We found that the glass column, which allowed the protein to contact the column surface during the injection procedure (i.e. in the sample loop), and the lower density column packing resolved the irreproducibility and attendant problems.

**Fluorescence Spectroscopy**—Fluorescence excitation and emission spectra were measured using an SLM Aminco-Bowman spectrophotofluorometer (SLM Instruments, Urbana, IL). Corrected excitation spectra were obtained directly using an internal rhodamine B standard; emission spectra were uncorrected. The fluorescence intensity was determined by photon counting, and the temperature of the cell was controlled to within ±0.1°C by use of a circulating water bath controlled by a thermostatted cell holder. All spectra were calibrated with a 2-×10-mm quartz fluorescence cell, oriented to enable the exciting beam to pass through the shorter cell path and thereby minimize the inner filter effect (25).

The extent of the inner filter effect using this cell was determined by analyzing the emission intensity of quinine sulfate in 0.1 N H₂SO₄, as a function of absorption at the excitation wavelength. To correct for any inner filter effect, the emission spectra were digitally multiplied by the appropriate factor determined from the quinine sulfate data. In practice, little inner filter effect was observed in samples in which the absorption intensity at the excitation wavelength was 0.8 absorbance unit or less in a 1-cm cell.

The enhancement of bound isocolchicine and colchicine fluorescence was assessed by the following procedure and analysis. The emission spectra of the ligands in the presence and absence of tubulin were first corrected for any inner filter effect using the calibration method described above. The emission spectra were then corrected for background fluorescence by digital subtraction of the spectrum of unbound ligand from the ligand/tubulin and the spectrum of PMG buffer from the spectra of unbound ligand. Then, the fluorescence intensity due to tubulin-bound ligand was determined by subtracting the corrected emission intensity of the ligand in the absence of tubulin from that of the ligand in the presence of tubulin. This calculation neglects the difference between the total and free ligand concentrations, which is small (usually 1% or less) due to the large excess of ligand used in each experiment.

To evaluate and compare the magnitude of the fluorescence enhancement of each ligand when bound to tubulin, the actual concentration of bound ligand must be determined. The actual concentration of bound ligand is obtained through the use of the following formulation which is derived from the Law of Mass Action:

\[
p = f (J_{f}T_{f} + 1 + 1/(K_{T}T_{f})) = L_{f}T_{f} = 0
\]  

where \( f \) = fraction of tubulin bound with isocolchicine, \( T_{f} = \) total concentration of tubulin, \( L_{f} = \) total concentration of ligand, and \( K \) = the ligand-tubulin association constant.

At each concentration of ligand and tubulin examined, the equation was solved for \( f \), the fraction of tubulin to which ligand is bound. The association constant for isocolchicine binding to tubulin was determined in this study; the association constant for colchicine binding to tubulin was taken to be 2.2 × 10⁵ M⁻¹(17). The value for \( f \) was multiplied by the total concentration of tubulin to yield the concentration of the complex. Finally, the corrected emission spectra were normalized to 1 μM bound species by multiplication by the appropriate factor.

**Gel Filtration Binding Assays**—The extent of isocolchicine binding to tubulin was assessed by the method of Hummel and Dreyer (26) as modified for use on a Spectra Physics 8700 HPLC system. The HPLC system was equipped with a Spectra Physics 4 fow restriction valve between the pump and the detector to supply sufficient back pressure for consistent, reproducible pump operation and with a Bio-Gel P-6 column (0.9 × 13 cm) which was equilibrated with filtered, degassed PMG buffer containing the chosen concentration of isocolchicine. This system is a modification of that reported by Williams et al. (27) who employed a Chromegapore MSE-100 column; we were unable to obtain reproducible results with the Chromegapore column, apparently due to substantial protein denaturation during the course of the experiment. We found that the glass column, which allowed the protein to contact the column surface during the injection procedure (i.e. in the sample loop), and the lower density column packing resolved the irreproducibility and attendant problems.

Tubulin in PMG buffer was mixed with isocolchicine to yield the same concentration of isocolchicine as in the equilibration buffer, and 50- to 100-μl samples were applied to the column through a Rheodyne injector. The flow rate was 1.0-1.5 ml/min. The effluent was monitored at 248 nm using an Iasco V-4 variable wavelength detector containing a preparative flow cell (5 mm path length, 10 μl illumination volume). The areas of the peak and trough were determined by integration using a Varian 4270 recording integrator. These could be related to the concentration of isocolchicine in the following manner. The Bio-Gel P-6 column was equilibrated with PMG buffer, 50-μl injections of solutions of known concentrations of isocolchicine were made, and the areas of these peaks were determined by electronic integration. The peak areas were corrected for the small absorbance of bound ligand at this wavelength. A flow rate of 1.5 ml/min, the peak and trough eluted at retention times of 2.6 and 6.5 min, respectively.

Precise measurements of the extent of ligand binding were made by determination of the trough areas which resulted when different amounts of isocolchicine were applied together with a constant concentration of isocolchicine (26). Plotting the areas of the trough vs. the moles of excess isocolchicine injected on the column and the concentration of isocolchicine in which the column is equilibrated resulted in a straight line which was used to calculate the moles excess needed to fill the trough (i.e. the molar equivalents of
isocolchicine bound to the protein). This technique required that the area of the peak at the void volume remain constant for various amounts of excess ligand (28). This condition was satisfied in the experiments presented here.

The association constant for isocolchicine-tubulin binding ($K$, Equation 4) was calculated from the concentration of isocolchicine bound to tubulin, determined as outlined above, using Equation 3 which can be derived assuming the equilibrium illustrated in Equation 4:

$$K = \frac{f(T_e) - f(T_b)}{(T_e - f(T_b))} \left[ I_0 - f(T_b) \right]$$

$$T + I = [T - I]$$

where $f = \text{moles of isocolchicine bound/mol of tubulin}$, $T_e = \text{total concentration of tubulin}$, and $I_0 = \text{concentration of isocolchicine in which the column was equilibrated}.$

Assays for the Competitive Binding of Isocolchicine and Colchicine—The ability of isocolchicine to inhibit the binding of $[^3H]$colchicine to tubulin was assessed by a modification of the filter disk assay routinely employed in investigations of the binding of colchicine analogs to the colchicine site on tubulin (17, 31), to provide greater ease and reproducibility in the separation of protein-bound $[^3H]$colchicine by scintillation spectrometry. Control experiments were performed to determine the levels of $[^3H]$colchicine in the effluent in the absence of tubulin (generally negligible) and the concentration of tubulin in the effluent. Although the procedure for separating the bound ligand differs, we have found that the $K_i$ values reported for these ligands using the filter disk procedure are virtually identical with those procedures occurring in the separation of protein-bound $[^3H]$colchicine by scintillation spectrometry. Control experiments were performed to determine the levels of $[^3H]$colchicine in the effluent of isocolchicine and $[^3H]$colchicine as previously described (17), then application of aliquots of the incubation mixtures (100 μl) to a prepared column (31), centrifugation for 2 min at 900 rpm in an IEC Centra 7 centrifuge, and analysis of the effluent for tubulin-bound $[^3H]$colchicine by scintillation spectrometry. Control experiments were performed to determine the levels of $[^3H]$colchicine in the effluent in the absence of tubulin (generally negligible) and the concentration of tubulin in the effluent. Although the procedure for separating the bound ligand differs, we have found that the $K_i$ values for alcochicine and 2-methoxy-5- (2',3',4'-trimethoxyphenyl) tropone determined using this procedure are virtually identical with the values reported for these ligands using the filter disk procedure.

Assays for the Inhibition of Microtubule Assembly—Tubulin polymerization was performed in PM buffer (100 mM Pipes, 1.0 mM MgSO$_4$, 2.0 mM EGTA, pH 6.9 at 23 °C) using a tubulin concentration of 2.0 ± 0.1 mg/ml. The tubulin and tubulin/isocolchicine solutions were cooled at 4 °C in a cuvette placed in a thermostatted cell holder. GTP was added to a concentration of 1.0 mM, and microtubule-associated proteins (MAPs) were added to produce 20% (w/w) of the tubulin. Assembly was initiated by rapidly raising the temperature to 37 °C, and the progress of tubulin polymerization was monitored by the increase in turbidity of the solution at 400 nm using a Cary 218 UV-vis spectrophotometer.

**RESULTS**

Investigation by UV Difference Spectroscopy of the Isocolchicine-Tubulin Interaction—The interaction of isocolchicine with tubulin was initially investigated by UV difference spectroscopy (Fig. 2). The difference spectrum was measured by varying concentrations of isocolchicine with the following spectral characteristics being consistently observed: a positive absorption band centered around 350 nm, a positive (shoulder) band at approximately 300 nm, and a small negative peak at approximately 280 nm. In addition, a large positive band was observed at around 250 nm, corresponding to the region of trimethoxyphenyl absorption in the UV spectrum of isocolchicine, although the signal/noise ratio was insufficient to enable measurement of the entire peak due to the large absorption of both tubulin and isocolchicine in this region.

As a control, the difference spectrum of 5 μM tubulin and 2 μM colchicine (i.e. 1% of the isocolchicine concentration of Fig. 2) was examined. After a 1-h incubation at 37 °C, the colchicine difference spectrum showed a pattern similar to the previously published spectra (11) (i.e. positive peaks at ~385 and ~365 nm, an inflection point at ~330 nm, and a negative peak at ~320 nm). The maximum increase in absorption was ~0.0025 absorbance unit at 385 nm. Thus, besides having a gross overall appearance unlike the colchicine difference spectrum, the absorption intensity observed in the isocolchicine difference spectrum is too large to be attributed to contaminating colchicine.

The observation of a difference spectrum between isocolchicine and tubulin indicates that an interaction has occurred between the ligand and protein which perturbs the UV spectrum of one or both of the agents. The data suggest that the spectra of both species are perturbed upon association: an apparent increase in the extinction coefficient was observed for the near-UV band for isocolchicine, although there was no detectable shift in the absorption maximum of this band, and an apparent decrease was observed in the extinction coefficient of the absorption spectrum of the protein at 280 nm, which corresponds to a trough in the isocolchicine spectrum (data not shown). Studies of the time dependence for appearance of a difference spectrum were undertaken at single wavelengths for several isocolchicine concentrations and at several temperatures. These investigations indicated that equilibrium was approached rapidly (e.g. faster than the cuvettes could be assayed after mixing; <10 s) for the isocolchicine-tubulin interaction.

The isocolchicine-tubulin interaction could not be quantified utilizing this technique due to the unacceptably large signal-to-noise ratio, despite considerable efforts employing a variety of ligand concentrations and incubation conditions.

![Fig. 2. The UV difference spectrum of isocolchicine and tubulin.](image-url)
Our subsequent investigations were directed at quantifying the isocolchicine-tubulin interaction and at determining the protein site for ligand association.

Determination of the Association Constant for the Isocolchicine-Tubulin Interaction—The association of isocolchicine and tubulin was quantitatively analyzed by the method of Hummel and Dreyer (26) modified for HPLC use as described under "Experimental Procedures." A representative Hummel-Dreyer experiment is presented in Fig. 3A, the presence of a peak and a trough in the chromatogram further demonstrating that isocolchicine binds to tubulin.

The extent of ligand-protein binding was established in one method by determination of the trough areas which resulted when different quantities of isocolchicine were applied together with a constant level of tubulin to a buffer containing a constant concentration of isocolchicine. The results of such an experiment are presented in Fig. 3B. The data were analyzed by plotting the molar excess of isocolchicine (positive or negative) versus the area of the resulting trough. When the area of the trough is equal to zero, an amount of excess ligand has been added which will exactly fill the trough. At this quantity, the number of moles of excess ligand is equal to the number of moles bound to the protein. From this value, the association constant for the interaction may be calculated assuming 1 binding site per tubulin dimer. At 23°C, an average value of 4.0 ± 1.5 × 10³ M⁻¹ was determined for the isocolchicine-tubulin interaction based on three independent experiments.

In an alternate method for calculation of the association constant, the areas of the peaks, which remained constant within a set of experiments, could be utilized to determine the amount of isocolchicine bound to tubulin as a function of the ligand concentration. Using this method of analysis, the association constant was determined to be 6.4 ± 0.9 × 10³ M⁻¹ based on four separate experiments. Thus, the average value determined from these two methods of analysis for the association of isocolchicine with tubulin is 5.5 ± 0.9 × 10³ M⁻¹.

That the association is due to isocolchicine and not contaminating colchicine can be determined by analyzing the data in Fig. 3B. When the isocolchicine concentration equals ~87 μM, the concentration of the ligand-tubulin complex is ~3 μM. If isocolchicine does not bind to tubulin, then the complex must be due to contaminating colchicine. Thus, the colchicine concentration would have to be ~3% of the total concentration of the isocolchicine solution. Since this concentration is well within our analytical limits, we conclude that the association measured with the Hummel-Dreyer method is that of isocolchicine and not colchicine binding to tubulin.

Investigation of the Competitive Binding to Tubulin of Isocolchicine and Colchicine—We next addressed the issue of whether the isocolchicine interaction with tubulin was a consequence of association at the colchicine binding site on tubulin. Data demonstrating the isocolchicine-dependent inhibition of [³H]colchicine binding to tubulin are presented in Fig. 4. These data demonstrate that isocolchicine weakly inhibits the binding of colchicine with a Kᵢ = ~400 μM, consistent with competitive binding to the colchicine site on tubulin.

Investigation of the Inhibition of Tubulin Polymerization by Isocolchicine—The binding of agents to the site on tubulin typically results in the inhibition of tubulin polymerization into microtubules. Results of studies undertaken to examine the inhibition of microtubule assembly by isocolchicine are presented in Fig. 5. Isocolchicine was found to inhibit tubulin polymerization.
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FIG. 5. The inhibition of microtubule assembly by isocolchicine. The tubulin concentration in all cases was 2.0 mg/ml, and MAP concentration was 0.2 mg/ml. Polymerization was initiated as described under “Experimental Procedures.” The number legends are: (1) no isocolchicine; (2) 0.5 mM isocolchicine; (3) 1.1 mM isocolchicine; (4) 2.6 mM isocolchicine.

FIG. 6. A, fluorescence emission spectra of isocolchicine (102 μM) and colchicine (0.21 μM) in the presence and absence of tubulin (9.6 μM). The concentrations of tubulin-bound isocolchicine and colchicine are ~3.4 μM and ~0.2 μM, respectively (calculated as described under “Experimental Procedures.”) The number legends are: (1) isocolchicine (102 μM) and tubulin (9.6 μM) in PMG buffer; (2) isocolchicine (102 μM) and tubulin (9.6 μM) in PMG buffer. The emission spectrum of 0.21 μM colchicine in PMG buffer coincides with that of the background; thus, a separate spectrum is not shown. The spectra were corrected for an inner filter effect (as described under “Experimental Procedures”), and backgrounds are subtracted. The excitation wavelength was 340 nm, and the excitation and emission slits were 16 nm and 8 nm, respectively. The temperature for all spectra was 37°C. B, relative fluorescence emission of isocolchicine (1) and colchicine (2) bound to tubulin. The spectra of the bound species from A were determined as described under “Experimental Procedures” and were normalized to 1 mM complex.

polymerization, and the concentration that led to 50% inhibition relative to the control value (= I50) was approximately 1.1 mM. Clearly, at the high concentrations used, there may be some dimerization of isocolchicine, as a 1 mM colchicine solution has been shown to contain ~10% dimerized colchicine (32). A similar association process for isocolchicine could contribute to the large I50 value observed for this compound. In contrast, 2 μM colchicine inhibited tubulin polymerization by 50% if the drug-tubulin solution was preincubated at room temperature for 30 min prior to initiation of polymerization.

Examination of the Fluorescence Spectroscopy of the Isocolchicine-Tubulin Complex—Since the binding of colchicine to tubulin results in a dramatic enhancement in the fluorescence of the tropone chromophore, we undertook an investigation of the fluorescence characteristics of the isocolchicine-tubulin complex. The emission spectra of isocolchicine in the presence and absence of tubulin are presented in Fig. 6A. Like colchicine, isocolchicine exhibits a weak fluorescence in aqueous solution (curve 1 in Fig. 6A), which is slightly enhanced in the presence of tubulin (curve 2 in Fig. 6A). The increase in tubulin-bound isocolchicine fluorescence, however, is much less than that seen for colchicine-bound tubulin (curve 3 in Fig. 6A). In order to compare the emission intensities of the bound ligand, the emission spectra of the tubulin-ligand solutions were corrected for background fluorescence and unbound ligand fluorescence, and the resulting emission spectra were normalized to reflect the relative amount of ligand-protein complexes in the solutions (see “Experimental Procedures”). The normalized emission spectra of the bound ligands are shown in Fig. 6B and demonstrate that the isocolchicine-tubulin complex is only very weakly fluorescent compared to tubulin-bound colchicine.

In order to ensure that the fluorescence observed is due to isocolchicine and not contaminating colchicine, we performed an experiment in which colchicine was added to an isocolchicine solution to a final concentration of 0.2% of the isocolchicine concentration. The fluorescence intensity of the “contaminated” solution in the presence of tubulin was ~4-fold greater than the intensity of the isocolchicine-tubulin sample (data not shown). We therefore conclude that the very weak fluorescence of isocolchicine in the presence of tubulin is most likely due to the association of isocolchicine and not contaminating colchicine with tubulin.

DISCUSSION

We have demonstrated that isocolchicine, a closely related structural isomer of colchicine, binds weakly to tubulin (K = 5.5 ± 1.5 × 10^5 M^-1 at 23°C) at the colchicine binding site of the protein (Kf = ~400 μM for the competitive inhibition of [3H]colchicine binding). The binding process is rapid, produces an inhibition of microtubule assembly (I50 = ~1 mM), and enhances ligand fluorescence, although not to the same extent as colchicine. These findings contrast with numerous previous investigations of the in vitro and in vivo pharmacology of isocolchicine, which suggested that isocolchicine was ineffective in the inhibition of microtubule assembly and was implicitly assumed not to bind to tubulin (17–20). The differences between the work reported here and prior studies are relative, however; isocolchicine does not possess therapeutically useful pharmacological indices, the target of most prior studies, being 200- to 500-fold less active than colchicine in the tubulin binding assays detailed above.

It is clear from the data that isocolchicine interacts with tubulin in a manner quite different from colchicine. The association is ~500-fold weaker, and the potency of isocolchicine in inhibiting microtubule assembly is reduced, when compared to colchicine, by approximately the same factor. The spectroscopic studies further demonstrate the differences in the isocolchicine- and colchicine-tubulin complexes. For example, isocolchicine fluorescence is only moderately enhanced when liganded to tubulin, and the absorption differ-
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1A

1B

2A

2B

Fig. 7. The conformations of colchicine (1A and 1B) and isocolchicine (2A and 2B) obtained by boat-boat interconversion of the tropone ring. The principal conformer of colchicine in solution is 1A and the conformer proposed to result in tight association with tubulin is 1B (6). Both conformers of isocolchicine possess virtual structural homology with the ground state conformation of colchicine (1A).

ence spectra do not display the bathochromism of the colchicine-tubulin complex. These observations point to an altered environment for the C ring portion of the two molecules in the complexed state.

One possible explanation for the differences in the association of the two drugs stems from a mechanism for colchicine binding to tubulin in which an initial ligand-protein complex undergoes conformational adjustments of both the drug and the protein (6). In this proposed mechanism, the ground state conformation of colchicine, which has a biaryl angle of ~54°, undergoes initial protein association. The resulting near-planar biaryl species, which is produced via boat-boat conformational interconversion of the methoxytropone moiety and has a biaryl angle of ~13°, then produces the "tight" ligand-protein complex in a process that involves concomitant protein conformational adjustment. We have previously suggested that the characteristic, distinct spectral features associated with the tropone chromophore produced upon colchicine-tubulin binding may, in a large measure, be a consequence of the near planar conformational state of colchicine and that the high activation energy of the process is due to the simultaneous conformational readjustment of ligand and protein.

Examination of the possible conformational behaviors of colchicine and isocolchicine illustrates a significant difference: fluxional interconversion of the tropone C ring of isocolchicine does not produce a conformer in which the trimethoxyphenyl A ring and troponoid C ring are nearly planar, as is the case with colchicine (Fig. 7). Instead, both low energy C ring conformations produce highly "skewed" biaryl A,C ring relationships (of ~45° and ~60°). In terms of the above mechanism, then, isocolchicine could associate with tubulin to form the initial, low affinity complex characteristic of the first step of the binding process. Indeed, the association constant determined for isocolchicine (5.5 ± 1.5 x 10^5 M^-1 at 23 °C) is very similar to the association constant that Garland (9) determined for the initial, low affinity complex of colchicine and tubulin (e.g. Kt = 8.1 ± 1.2 x 10^5 M^-1 at 37 °C for [TC] in Equation 1). The inability of isocolchicine to adopt the second, more planar conformation analogous to colchicine would rationalize the more rapid binding as well, since the high activation energy for the formation of the tubulin-colchicine complex is a consequence of the second step of the binding. Furthermore, the relatively small alterations in the absorption and fluorescence properties of tubulin-bound isocolchicine would be anticipated, as the environment but not the conformational state of isocolchicine is altered upon tubulin binding.

Alternatively, it is possible that the differences in isocolchicine and colchicine binding to tubulin are the result of the inversion of the C-9 carbonyl and C-10 methoxy moieties without regard to possible alternate conformational states of either molecule. Several investigations have indicated that the biaryl moieties of colchicine, consisting of the A and C
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rings, is the essential pharmacophore for tubulin binding (33, 34). Isosteric substitutions of the C-10 substituent of colchicine (with the exception of -OH3) have been shown to have little impact on the association properties of these ligands with tubulin (35). When the size of the C-10 substituent is greatly altered, however, substantial decreases in drug potency is observed. Increased steric bulk greatly decreases activity (36), while elimination of the C-10 substituent essentially abolishes tubulin binding (37). It is presently unclear what properties of the C-10 substituent (i.e., steric, electronic, or a combination of both) contribute to colchicinoid-tubulin complex stability. We are currently engaged in exploring the role of the C-10 substituent in some detail, results that will assist in unraveling the puzzling nature of the isocolchicine-tubulin complex.

In the absence of an altered conformational state of tubulin-bound colchicinoids, the unique spectral features of the colchicine-tubulin complex would be the result of a specific association between the ligand and the protein and not solely due to the relationship between the A and C ring chromophores in the bound species. Bhattacharyya and Wolff (19) performed an elegant fluorescence study and proposed that the fluorescence enhancement of tubulin-bound colchicine could be due in part to immobilization of the ligand upon complex formation. Fluorescence of isocolchicine in the presence of tubulin was not observed, presumably because of the relatively large concentrations of isocolchicine required for binding and the high inner filter effects which are encountered when standard fluorescence cells are utilized. According to this proposal for the origin of tubulin-induced fluorescence enhancement, isocolchicine may not be held as "rigidly" as colchicine in the receptor site, and the relatively low quantum yield of the isocolchicine-tubulin complex may be mostly attributable to an environment of decreased dielectric constant.

The association of colchicine with tubulin is an important interaction with regard to therapy and has spawned the medicinal chemical development of over 200 analogs (35, 38). Yet, an understanding of the molecular mechanisms of drug binding to this site remains elusive. In this paper, we have presented evidence that isocolchicine, previously believed not to bind to tubulin, associates weakly with the colchicine receptor site. The data are consistent with a mechanism for colchicinoid binding to tubulin which involves a conformational interconversion of both the protein and ligand after initial ligand-protein association, but it is possible to rationalize some of the observations without proposing an altered conformational state of the tubulin-bound ligand. In order to resolve the mechanistic uncertainties, direct assessment of the conformational and electronic properties of tubulin-bound colchicinoids have been initiated using more sensitive spectroscopic techniques (39). In the meantime, the studies presented here further delineate the ligand structural requirements for the colchicine receptor site on tubulin.

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