

Interaction of colchicine analogues with purified tubulin

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Binding of two colchicine analogues, desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone to purified tubulin have been studied. Both analogues bind to tubulin with a significant increase in fluorescence polarization of the drugs in solutions containing tubulin. The K_d for tubulin-drug complexes were found to be 1.25×10^{-6} M and 1.08×10^{-6} M for desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone, respectively. Scatchard analysis of the fluorescence titration curve of drug tubulin interaction also gives the values of stoichiometry and affinity constant. These were 0.8 and 1.6×10^6 M⁻¹ for desacetamidocolchicine, and 0.9 and 0.58×10^6 M⁻¹ for 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone.

Brain tubulin B-ring of colchicine Fluorescence Polarization Binding parameter

1. INTRODUCTION

Colchicine binds to native tubulin with stoichiometry of 1 mol alkaloid per mol tubulin dimer. Several features of this colchicine binding reaction are unusual and the question arose whether or not they were a property of colchicine or of the binding site. The rates of association and dissociation of colchicine and tubulin are slow: the activation energy for the binding reaction is about 20 kcal/mol [1]. Although no direct proof exists, there are evidences which indicate that this slow rate of colchicine binding reflects a ligand-induced conformational change in tubulin molecule [2-5]. Recently, authors in [6] presented circular dichroic measurements indicating that colchicine itself undergoes a change in conformation upon binding to tubulin. We have recently shown that the slow association rate of colchicine is a phenomenon of the B-ring substituent of colchicine molecule [7,8]. Inspection of the structures of the B-ring analogues of colchi-

cine (fig.1) reveals that, unlike colchicine and desacetamidocolchicine, the fluorophore (tropone moiety) of 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone can assume an infinity of conformations due to free rotation around the single bond between A and C-rings. It seems likely that

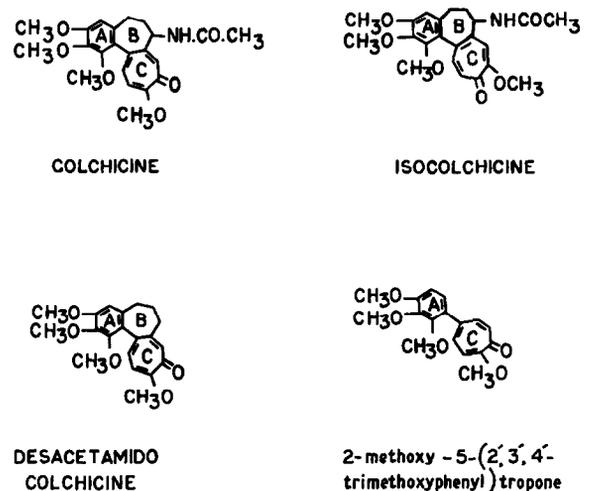


Fig.1. Structure of colchicine and some of its analogues.

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the active conformation would be similar to that of colchicine, since isocolchicine (fig.1) has no tubulin binding ability [9]. In fact, colchicine and isocolchicine can be considered as semi-rigid analogues of bicyclic compound. The rigidity in colchicine and desacetamidocolchicine molecule is imparted by the B-ring which anchored the A and C-ring. Thus it was anticipated that the fluorescence polarization which depends on the size, molecular geometry and rigidity of a molecule might be different for desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone when they bind with tubulin. Here some important binding properties of these two drugs with tubulin have been reported.

2. MATERIALS AND METHODS

Tubulin was prepared from goat brain according to [10]. The two cycles pellet was kept in -70°C and tubulin was further purified by phosphocellulose chromatography [11]. The purity of the tubulin was checked by SDS-gel electrophoresis [12]. The concentration of protein was determined as in [13] using bovine serum albumin as standard.

Fluorescence was measured in a Perkin-Elmer MPF 44B fluorescent spectrophotometer. Temperature was controlled with LKB 2209 Multitemp water circulator. The excitation and emission wavelengths were 350 nm and 430 nm, respectively. Polarization was done in the same spectrofluorometer equipped with the polarizing accessories.

2.1. Polarization

Polarization, P , of a molecule is expressed quantitatively in terms of intensities, I , polarized either parallel (\parallel) or perpendicular (\perp) to the incident electric field and can be calculated according to the equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

2.2. Scatchard analysis

- r = mol of drug bound per mol of tubulin
= $(F_c/F_o)/(P_o/C_o)$, where
- F_c = the fluorescence of a given solution of drug-tubulin complex;
- F_o = the fluorescence of an equal concentration of drug in excess tubulin, such that all the drug is bound;

C_o = the total drug concentration;
 P_o = is the total protein concentration.

From the known total concentration of drug and tubulin and the measured value of r , the free colchicine concentration, C , is calculated from the relation $C = C_o - rP_o$.

All binding experiments were carried out in buffer A of pH 6.4 with the following composition: 25 mM 2-(*N*-morpholino) ethane sulfonic acid (MES), 1 mM 2-mercaptoethanol, 0.5 mM Mg^{2+} , 0.1 mM GTP. Phosphocellulose (P11) was obtained from Whatmann. MES and GTP were from Sigma and other chemicals were of analytical grades.

3. RESULTS

It has been reported earlier that colchicine has no intrinsic fluorescence, however, induction of

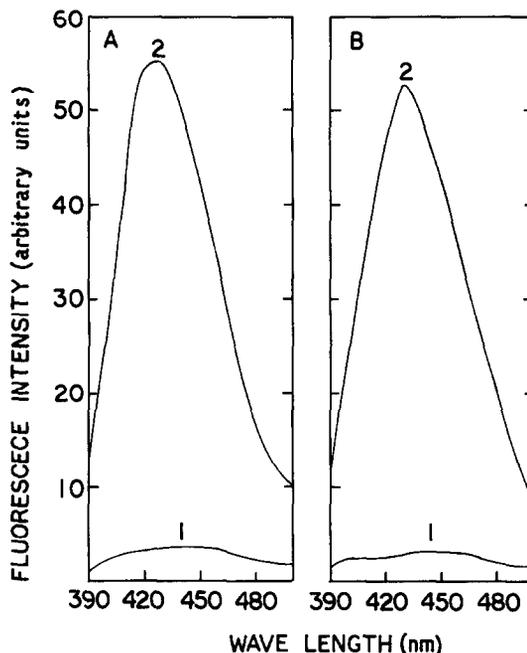


Fig.2. Fluorescence emission spectra of the drug-tubulin complex. Mixture of $14.5 \mu\text{M}$ tubulin and $10 \mu\text{M}$ drug in buffer A was incubated at 37°C for 30 min. The excitation wavelength was 350 nm. Panel A, desacetamidocolchicine: 1, free drug; 2, drug-tubulin complex; Panel B, 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone: 1, free drug; 2, drug-tubulin complex.

fluorescence occurs when the drug binds to tubulin [9]. Compared to colchicine its two structural analogues desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone have slight fluorescence in buffer A which enhanced in the presence of tubulin. The fluorescence emission spectra of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone are shown (fig.2). Like colchicine, both analogues have emission maxima at 430 nm when combined with tubulin. In the free state both drugs have emission maxima at around 405 nm and increase in the hydrophobic character of the solvents (using different organic solvents) do not lead to any enhancement in fluorescence.

Polarization of a molecule depends on its size, molecular geometry and rigidity. Tropone moiety (ring-C) responsible for the colchicine fluorescence is highly rigid in desacetamidocolchicine whereas it can assume an infinity of conformations by free rotations around the single bond between A and C-ring in the case of 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone.

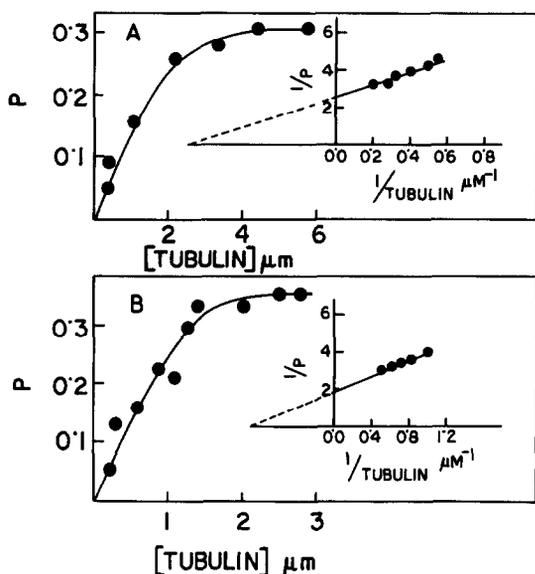


Fig.3. Titration of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone with purified tubulin in buffer A. Aliquots of tubulin were incubated with 5×10^{-7} M drug for 30 min. The polarization was measured as described in section 2 for each concentration of tubulin. Inset: Double reciprocal plot of the data.

(A) Desacetamidocolchicine; (B) 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone.

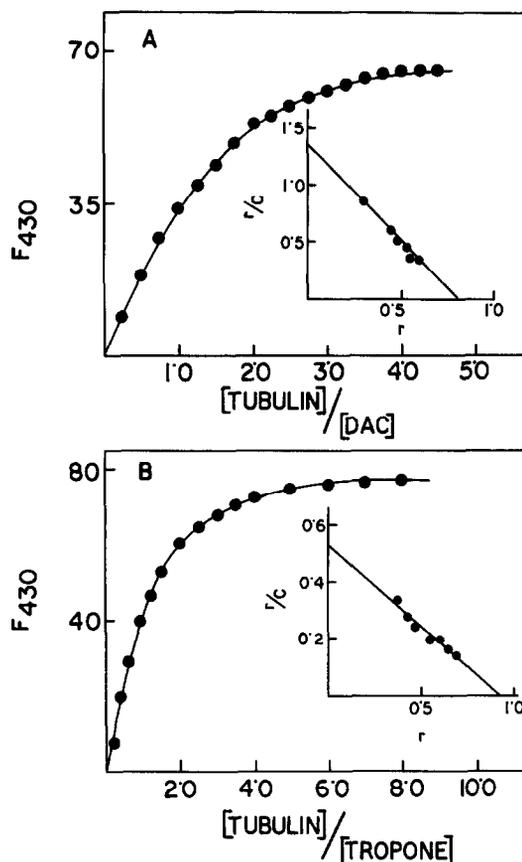


Fig.4. Binding parameters of the drug-tubulin complex. (A) Desacetamidocolchicine-tubulin fluorescence at 430 nm (F_{430}) as a function of increasing amounts of tubulin in buffer A. 2 μ M drug, incubated at 37°C for 30 min. Inset: Scatchard analysis of the data, calculated as described in section 2. (B) 2-Methoxy-5-(2',3',4'-trimethoxyphenyl) tropone-tubulin fluorescence at 430 nm (F_{430}) as a function of increasing amounts of tubulin in buffer A. 5 μ M drug, incubated at 37°C for 30 min. Inset: Scatchard analysis of the data, calculated as described in section 2.

oxygenophenyl) tropone. However, the polarization of both drugs are nearly zero in free state. Binding to tubulin however accompanied by a significant increase in the polarization of fluorescence of both desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone. Increase in the fluorescence polarization of desacetamidocolchicine (5×10^{-7} M) and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (5×10^{-7} M) with increase in tubulin concentrations are shown (fig.3). From

this typical binding equilibrium, the number of binding parameters of drug tubulin interactions are determined by means of double reciprocal plots. Plot of $1/\text{polarization}$ vs $1/[\text{tubulin}]$ for desacetamidocolchicine (inset fig.3A) and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone (inset fig.3B) are shown. The intercept of such a plot is $1/P_{\text{max}}$ and the slope is equal to K_d/P_{max} when K_d is the dissociation constant for the tubulin-drug complex. Thus the dissociation constants calculated for desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone are 1.25×10^{-6} M and 1.08×10^{-6} M, respectively.

The tubulin-induced fluorescence of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone were also used to determine the affinity constants and the stoichiometries of the drug-tubulin complex. The number of binding sites were determined from the fluorescence titration curves (fig.4), from a plot of r/C against r (fig.4, insets). The affinity constants are $1.6 \times 10^6 \text{ M}^{-1}$ for desacetamidocolchicine and $0.58 \times 10^6 \text{ M}^{-1}$ for 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone. Stoichiometries are 0.8 for desacetamidocolchicine and 0.9 for 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone. Thus the values of binding parameters determined by two different methods are comparable.

4. DISCUSSION

Importance of B-ring of colchicine in its binding to tubulin is emphasized recently from this laboratory. It was reported that two important unusual properties of colchicine-tubulin interaction such as slow association rate (which needs long incubation time at 37°C) and the high temperature coefficient are phenomena of the B-ring substituents of colchicine [7]. Thus, colchicine analogues with smaller (colcemid) or no substituents in the B-ring (desacetamidocolchicine) bind tubulin remarkably faster than the colchicine [7,8]. A compound without the B-ring (2-methoxy-5-[2',3',4'-trimethoxyphenyl] tropone) binds tubulin at 4°C and the binding is almost instantaneous at 37°C. Although it is known that these two important structural analogues of colchicine bind to tubulin at the same site where colchicine binds and inhibits *in vivo* and *in vitro* polymerization of tubulin [7,14], this paper documents for the first time the important

binding parameters such as affinity constant and stoichiometry of those drugs with tubulin.

Due to their small size the low molecular mass compounds will give rise to a depolarized fluorescence and therefore, an increase in the polarization of colchicine analogues binding to tubulin was anticipated. Very similar polarization values of both desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone in the presence of tubulin indicate that the tropolone moiety in both compounds are equally rigid in spite of the absence of B-ring in the latter. Appreciable changes in the fluorescence polarization of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone as a consequence of their binding to tubulin have been utilized for measuring the binding parameters of their interaction.

Since the fluorescence of desacetamidocolchicine and 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone enhanced significantly in the presence of tubulin, this also provides an opportunity to compare the binding parameters obtained by fluorescence enhancement. The affinity constants determined by fluorescence polarization is in agreement with the values determined by fluorescence enhancement.

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