Oxalone and Lactone Moieties of Podophyllotoxin Exhibit Properties of Both the B and C Rings of Colchicine in Its Binding with Tubulin†

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ABSTRACT: Thermodynamics of podophyllotoxin binding to tubulin and its multiple points of attachment with tubulin has been studied in detail using isothermal titration calorimetry. The calorimetric enthalpy of the association of podophyllotoxin with tubulin is negative and occurs with a negative heat capacity change ($\Delta C_p = -2.47$ kJ mol^{-1} K^{-1}). The binding is unique with a simultaneous participation of both hydrophobic and hydrogen-bonding forces with unfavorable negative entropic contribution at higher temperature, favored with an enthalpy−entropy compensation. Interestingly, the binding of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC, a colchicine analogue without the B ring) with tubulin is enthalpy-favored. However, the podophyllotoxin−tubulin association depending upon the temperature of the reaction has a favorable entropic and enthalpic component, which resembles both B- and C-ring properties of colchicine. On the basis of the crystal structure of the podophyllotoxin−tubulin complex, distance calculations have indicated a possible interaction between threonine 179 of α-tubulin and the hydroxy group on the D ring of podophyllotoxin. To confirm the involvement of the oxalone moiety as well as the lactone ring of podophyllotoxin in tubulin binding, analogues of podophyllotoxin are synthesized with methoxy substitution at the 4′ position of ring D along with its isomer and another analogue epimerized at ring E. From these results, involvement of oxalone as well as the lactone ring of the drug in a specific orientation inclusive of ring A is indicated for podophyllotoxin−tubulin binding. Therefore, podophyllotoxin, like colchicine, behaves as a bifunctional ligand having properties of both the B and C rings of colchicine by making more than one point of attachment with the protein tubulin.

Podophyllotoxin, a competitor for the colchicine-binding site on tubulin, possesses antimicrotubular activity and has been known for decades from its first application in folk medicines to its most recent development in VP-16 as a DNA topoisomerase-II inhibitor (*1*). The structure of podophyllotoxin consists of a five-ringed system (A, B, C, D, and E), where ring A is a trimethoxy phenyl ring resembling that of colchicine (Figure 1). As a result, podophyllotoxin because of its partial structural similarities with colchicine acts as a competitive inhibitor of colchicine binding (*2*).

Concerning colchicine, previous studies involving different colchicine-site analogues have established beyond a doubt that the drug makes at least two points of attachment with tubulin through its A and C rings. For example, the trimethoxyphenyl ring (ring A) has been shown to be involved in tubulin binding because colchicine analogues with bulky substituents in the A ring (colchicoside) are unable to inhibit podophyllotoxin−tubulin binding (*3*). Similarly, analogues having an identical A ring but a modified C ring such as isocolchicine and lumicolchicine are biologically inactive and bind tubulin with a lower affinity (*4*). This has been substantiated by the fact that individual ring compounds, such as mescaline (an A-ring analogue) and methoxy tropone (a C-ring analogue), both bind tubulin with a lower affinity, as compared to colchicine (*5*). Recently, studies from our laboratory have shown that isocolchicine becomes biologically active, binds tubulin with a higher affinity, inhibits tubulin self-assembly at a low drug concentration, and competes with [^{3}H]-colchicine for binding to tubulin upon introducing suitable hydrophobic groups such as NBD or a dansyl moiety at the C-7 position of colchicine (*6*, *7*). These studies further demonstrate that two points of attachment of the drug with tubulin are essential for higher compound binding affinity and have confirmed the previous hypothesis that the B-ring side chain of colchicine also makes contact with tubulin and contributes toward drug-binding affinity (*8*).

The study presented herein was intended to determine whether podophyllotoxin also behaves as a bifunctional ligand through attachment of its A ring along with its tetralin (C and D rings), oxalone (D ring), and lactone (E ring) rings with tubulin. Concerning the tetralin and lactone moieties, if they are involved in tubulin binding, then the question becomes whether this part of the molecule behaves like the B or C ring of colchicine. The binding properties of the analogue having only A and C rings [2-methoxy-5-(2′,3′,4′-

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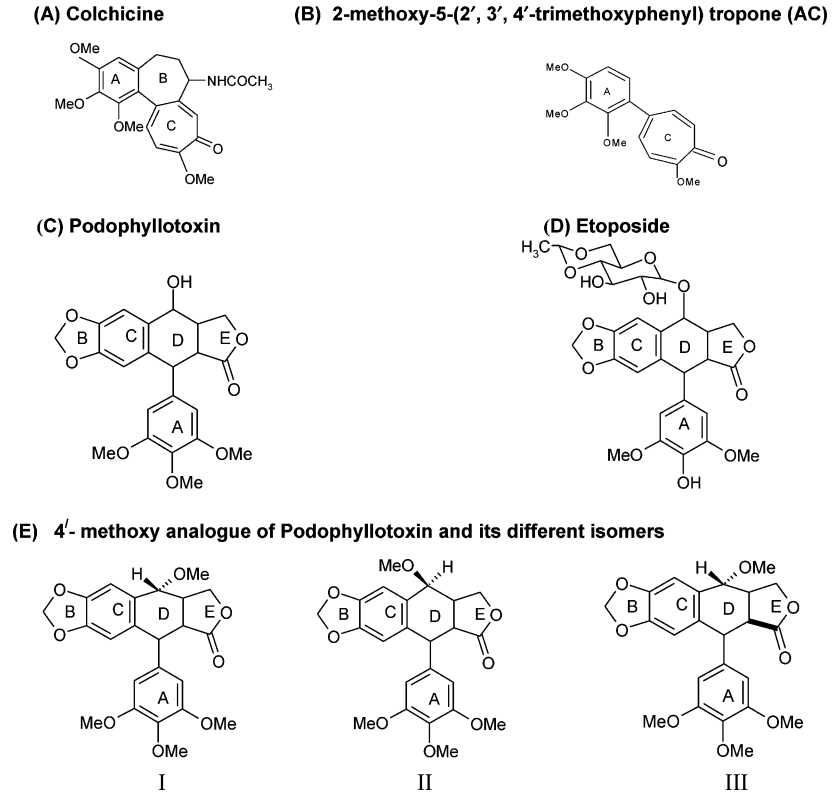

FIGURE 1: Structure of drugs. (A) Colchicine. (B) AC. (C) Podophyllotoxin. (D) Etoposide. (E) 4′-Methoxy analogue of podophyllotoxin and its different isomers.

trimethoxyphenyl)tropone] (AC)[1] (Figure 1) is distinctly different from that of colchicine. The AC−tubulin binding interaction is fast compared to colchicine (even at 0 °C), reversible, pH-independent, and thermodynamically governed by an enthalpic factor (*4*). In contrast, B-ring-modified colchicine analogues bind with tubulin slower (at 37 °C) and are poorly reversible, pH-dependent, and entropy-driven (*9*). Previous studies have also shown that podophyllotoxin−tubulin binding is pH-dependent (*3*). In addition, the structure of podophyllotoxin resembles that of AC in that both compounds possess a trimethoxy benzene ring connected to the remainder of the molecule through a single bond.

The thermodynamic parameters of the podophyllotoxin−tubulin interaction were previously reported using equilibrium methods (*3*). However, the determination of thermodynamic parameters using calorimetry is a more direct and straightforward method. Unfortunately, one of the major difficulties encountered with calorimetric titration as seen in the colchicine−tubulin interaction is the slow time-dependent binding of colchicine to tubulin. As such, the return to the baseline of the reaction after each injection is difficult, which can result in the loss of isothermal conditions during the titration. In the present study, however, such thermodynamic parameters as the change in enthalpy, entropy, free energy, and heat capacity of the podophyllotoxin−tubulin interaction were carefully determined calorimetrically and compared with similar parameters for the AC−tubulin interaction. This was done through the calorimetric titration of tubulin with podophyllotoxin at four different temperatures within the range of 25−37 °C in PEM buffer at pH 7. The enthalpy changes are negative within the range of temperatures studied and decrease linearly with temperature, resulting in a large negative value of change in the heat capacity, ΔC_p ($\Delta C_p = -2.47$ kJ mol^{-1} K^{-1}). This negative ΔC_p is an outcome of a hydrophobic interaction predicting the role of water as a mediator in the podophyllotoxin−tubulin interaction. The entropy change ΔS values obtained at relatively higher temperatures (33 and 37 °C) are negative, which reveals that the reaction is enthalpy-driven at higher temperatures. In addition, a enthalpy−entropy compensation plot observed for the podophyllotoxin−tubulin association deciphers a hydrophobic interaction as the major driving force involved in their binding (*10*). In contrast, for AC, the ΔC_p value is lower (−0.29 kJ mol^{-1} K^{-1}) and the reaction is favored by an enthalpy factor.

Previously, several cytotoxicity studies with different podophyllotoxin analogues have established that rings B, C, D, and A are involved in the binding reaction with tubulin (*11*). However, binding or thermodynamic studies were not performed with any of these analogues. Therefore, in this work, we have taken the 4′-methoxy derivative of podophyllotoxin along with its different isomers (Figure 1) and etoposide and elucidated their potency toward inhibition of tubulin polymerization as well as their binding with tubulin using isothermal titration calorimetry (ITC). We found that the isomers of podophyllotoxin are not equally effective in binding with tubulin. The prime determining factors for podophyllotoxin−tubulin bindings are the stereochemical orientation and the bulkiness of the substituent on ring D along with the orientation of ring E.

In conclusion, from this study, we have determined that podophyllotoxin like colchicine makes more than one point

[1] Abbreviations: AC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone; ITC, isothermal titration calorimetry; SAR, structure−activity relationship; PDB, Protein Data Bank; ASA, accessible surface area.

of attachment with tubulin. Furthermore, the thermodynamic parameters and the binding properties of the podophyllotoxin−tubulin interaction resemble that of AC as well as colchicine analogues with B-ring side chain at the C-7 position.

EXPERIMENTAL PROCEDURES

Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), guanosine 5′-triphosphate (GTP), ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), colchicine, and podophyllotoxin were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Synthesis of Podophyllotoxin Analogues. Podophyllotoxin analogues were synthesized as follows. The 4′-methoxy derivative of podophyllotoxin (analogue I) along with its isomer (analogue II) were synthesized by stirring podophyllotoxin with 1.1 equiv of trimethyloxonium tetrafluoroborate in CH_2Cl_2 at room temperature for 4 h. The reaction was then worked up, and the resulting isomers were separated using radial chromatography. The synthesis of the 4′-methoxy derivative of picropodophyllotoxin (analogue III) was as follows. Podophyllotoxin was dissolved in CH_3CN, and to this solution was added 2 equiv of Ag_2O along with 1.2 equiv of methyl iodide. The resulting solution was stirred at room temperature for 24 h, and the solution was then evaporated to yield a white solid. The solid was purified using radial chromatography to yield pure analogue III. The purity and identity of the compounds were determined using ^{1}H NMR and TLC analysis.

A stock solution was made with 100% dimethylsulfoxie (DMSO), and the drug concentration was determined from the extinction coefficients of 1.6694×10^4 and 1.884×10^4 M^{-1} cm^{-1} at 290 and 341 nm for the podophyllotoxin analogues and AC, respectively.

Tubulin Preparation and Estimation. Tubulin was isolated from goat brain by two cycles of temperature-dependent assembly and disassembly in PEM buffer (50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 6.9), in the presence of 0.1 mM GTP, followed by two additional cycles in 1 M glutamate buffer (*12*). The purified tubulin, free of MAPs, was stored in aliquots at −70 °C. Protein concentration was estimated by the method of Lowry et al. (*13*), using bovine serum albumin as the standard. Tubulin preparations used in this study contained a natural mixture of isoforms (*14*). Both calorimetry and fluorescence measurements were carried out with this unfractionated tubulin, and therefore, the binding parameters obtained here are averages for the different isoforms.

Calorimetry. Isothermal titration calorimetric measurements were performed on a VP-ITC MicroCalorimeter of MicroCal, Inc. (Northampton, MA). Tubulin was dialyzed extensively against PEM buffer with GDP (to offer stabilization), and the ligands (podophyllotoxin and AC) were dissolved in the last dializant. The pH values of the tubulin and the ligand solutions were made identical before loading into the calorimeter. A typical titration involved 25 injections of ligand (10 μL aliquots/shot), at 3 min intervals, into the sample cell (volume of 1.4359 mL) containing tubulin. The titration cell was kept at a definite temperature and stirred continuously at 310 rpm. The heat of dilution of the ligand in the buffer alone was subtracted from the titration data. The data were then analyzed to determine the binding stoichiometry (N), affinity constant (K_a), and thermodynamic parameters of the reaction, using Origin 5.0 software.

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 0.1 mM GTP. Polymerization was initiated using 10% dimethyl sulfoxide (Me_2SO), and the turbidity was measured by the absorbance at 360 nm. 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.

Binding Measurements by the Fluorescence Method. The binding of the ligands to the protein was monitored by enhancement of colchicine 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. Excitation and emission wavelengths used for the measurements were 350 and 430 nm, respectively. Both the excitation and emission bandpass was 5 nm in all cases.

Modified Dixon plots of the 4′-methoxy podophyllotoxin analogue I was obtained using colchicine as a competitive inhibitor. The reaction mixtures containing tubulin (3 μM) and varied concentrations of colchicine (5−15 μM) and the drug (0−30 μM) were incubated at 37 °C for 45 min. The reciprocal of the fluorescence intensity of the colchicine−tubulin complex at 430 nm was plotted against the concentration of the drug. The resulting Dixon plot gave an approximate K_i value for the analogue I.

RESULTS

Thermodynamics of Podophyllotoxin−Tubulin and AC−Tubulin Interactions. Figure 2A shows the raw data of a calorimetric experiment, which involved the titration of tubulin with podophyllotoxin in PEM buffer at 30 °C. As is evident from the figure, the binding reaction is characterized by a significant heat change. Figure 2B represents the enthalpy change upon binding for each injection as a function of the concentration of podophyllotoxin. The thermodynamic parameters ΔG, ΔH, and ΔS are determined over a range of temperatures from 25 to 37 °C and are presented in Table 1. The raw data of calorimetric titration of AC binding to tubulin is shown in Figure 2C. Figure 2D reveals the enthalpy change (ΔH) upon binding as a function of the concentration of AC for each injection. In Table 2, the values of the thermodynamic parameters are shown. There is almost no temperature dependence of the binding energy ($\Delta G = 0$) within the temperature range of 25−37 °C. This is due to the reciprocal contribution from the enthalpic and entropic components to ΔG (Table 2). The temperature dependence of the entropy and enthalpy change for the podophyllotoxin and AC binding to tubulin has been presented in Figure 3. The unfavorable negative value of ΔS at 37 °C becomes a positive value at lower temperatures for the podophyllotoxin−tubulin interaction (Figure 3A). The heat capacity change at constant pressure (ΔC_p) is determined using Kirchoffs equation as $d\Delta H/dT = \Delta C_p$. A plot of the enthalpy

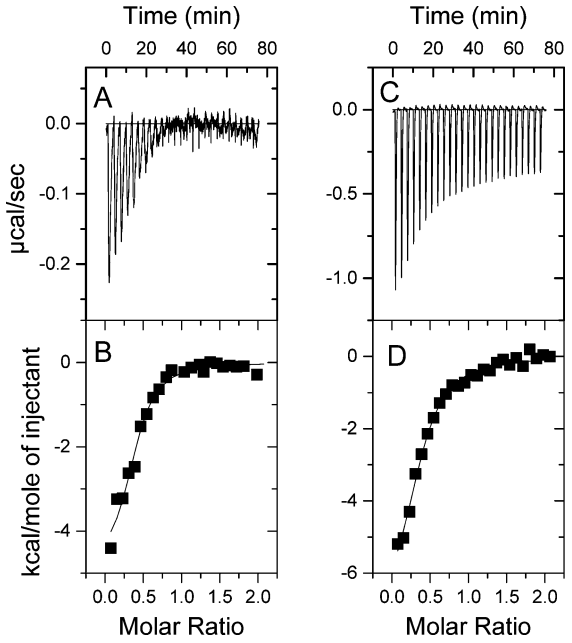

FIGURE 2: Calorimetric titration of tubulin with podophyllotoxin. (A) Raw data obtained from 25 injections of 10 μL aliquots of podophyllotoxin into 0.020 mM tubulin in 50 mM PIPES buffer (pH 7.0). (B) Nonlinear least-squares fit of the incremental heat per mole of added ligand for the titration in A by the injection number as a function of the molar ratio using Origin. Calorimetric titration of tubulin with AC. (C) Raw data obtained from 25 injections of 10 μL aliquots of AC into 0.020 mM tubulin in 50 mM PIPES buffer (pH 7.0). (D) Nonlinear least-squares fit of the incremental heat per mole of added ligand for the titration in C by the injection number as a function of the molar ratio using Origin.

Table 1: Thermodynamics of Tubulin−Podophyllotoxin Binding

temperature (K)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})	$T\Delta S$ (kJ/mol)	ΔC_p (kJ mol^{-1} K^{-1})
298	−33.70	−11.58	74.21	92.88	−2.47
303	−32.60	−20.63	39.51	50.28	
306	−33.16	−34.77	−5.27	−6.77	
310	−33.55	−39.15	−18.05	−23.50	

Table 2: Thermodynamics of Tubulin−AC Binding

temperature (K)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})	$T\Delta S$ (kJ/mol)	ΔC_p (kJ mol^{-1} K^{-1})
293	−30.76	−22.68	27.60	8.086	−0.289
298	−30.67	−23.25	24.92	7.42	
300	−31.04	−26.37	15.57	4.66	
306	−30.76	−26.09	15.26	4.67	

change (ΔH) for podophyllotoxin−tubulin binding as a function of temperature yields $\Delta C_p = -2.47$ kJ mol^{-1} K^{-1} (Figure 3B). It is evident from these experimental results that the reaction of AC with tubulin is enthalpy-driven with a significantly less negative ΔC_p value (−0.29 kJ mol^{-1} K^{-1}) compared to podophyllotoxin. The significantly lower ΔC_p value observed for AC indicates a lesser extent of surface−surface association between the protein−drug interfaces and the predominant involvement of hydrogen bonding during the reaction.

Enthalpy−Entropy Compensation of the Podophyllotoxin−Tubulin Interaction. A negative ΔC_p is a characteristic feature of a enthalpy−entropy compensatory effect, indicating water uptake or release upon burial of the nonpolar surface area (*15*). This is manifested by the decrease or increase in ΔS

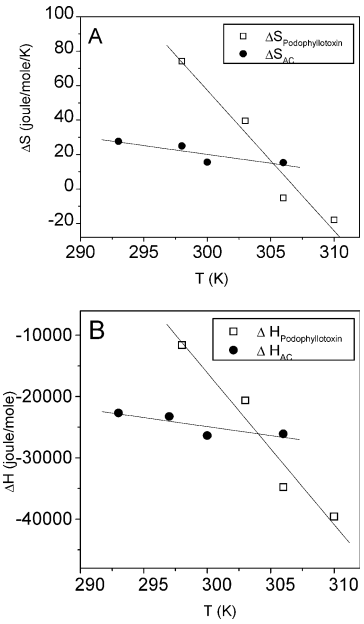

FIGURE 3: (A) Temperature dependence of the entropy change (ΔS) for podophyllotoxin (□) and AC (●) binding to tubulin. (B) Temperature dependence of the enthalpy change (ΔH) upon binding of podophyllotoxin (□) and AC (●) to tubulin at pH 7.0. The continuous line is the least-squares fit of the data.

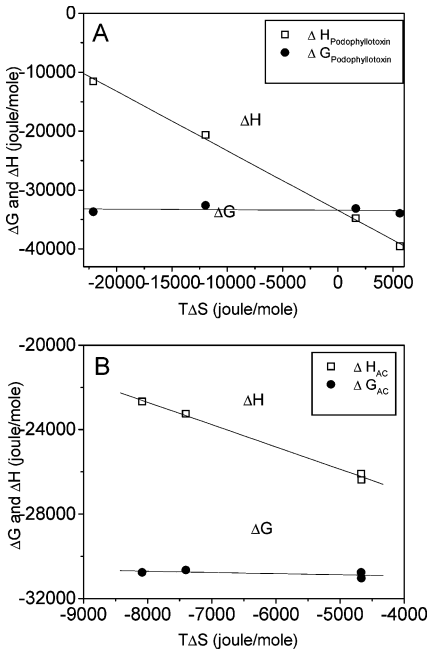

FIGURE 4: (A) Enthalpy−entropy compensation plot of ΔG (●) and ΔH (□) as a function of $T\Delta S$ for the binding of podophyllotoxin to tubulin. (B) Enthalpy−entropy compensation plot of ΔG (●) and ΔH (□) as a function of $T\Delta S$ for the binding of AC to tubulin.

values within the range of temperatures studied. An enthalpy−entropy compensation plot in Figure 4, where ΔH and ΔG are plotted against $T\Delta S$, shows a linear dependence of ΔH on $T\Delta S$. The contribution of hydrophobic effect is evident from the plot for the podophyllotoxin−tubulin interaction that has a slope close to 1 (Figure 4A). From previous studies, it is known that an exact compensation of enthalpy by entropy results in a slope close to 1.0 (*16*). Any deviation from this value suggests a different thermodynamic aspect of binding. A slope less than 1 is suggestive of a

Table 3: Hydrophobic and Vibrational Contribution to ΔC_p and ΔS for Podophyllotoxin−Tubulin Binding

temperature (K)	ΔS (J mol^{-1} K^{-1})	ΔS_{hydro} (J mol^{-1} K^{-1})	$\Delta S_{vibration}$ (J mol^{-1} K^{-1})	$\Delta C_{p_{hydrophobic}}$ (J mol^{-1} K^{-1})	$\Delta C_{p_{vibration}}$ (J mol^{-1} K^{-1})
298	74.21	530.25	−456.03	−2039	−434.28
303	39.51	523.36	−483.84	−2012	−460.78
306	−5.27	514.50	−519.75	−1978	−494.97
310	−18.05	511.98	−530.04	−1969	−504.75

predominant entropic contribution in the binding process (*17*). In contrast, a slope greater than 1 is indicative of a predominantly enthalpic binding process. For the AC−tubulin interaction, a slope greater than 1 (1.057) reflects an enthalpic contribution in the binding process (Figure 4B). The enthalpy−entropy compensation effect has been observed between the initial and final steps of colchicine and its analogues binding to tubulin (*18*). The values of the enthalpy change for the initial binding step (ΔH_1^0) occupy a wide range of values mostly endothermic for different colchicine analogues, but the enthalpy change for the final binding step (ΔH_2^0) follows a reverse trend and becomes exothermic. It was suggested that the initial binding promotes stability at the expense of the second step. The similar compensation effect has also been reported for molecules sharing very little or no structural resemblance with colchicine but compete with it for the same site on tubulin (*19*−*21*).

The negative ΔC_p causes the net thermodynamic driving force for the reaction to shift from entropic to enthalpic with increasing temperature. Extension of Baldwin's terminology (*22*) indicates the presence of two characteristic temperatures, namely, T_s and T_H within the range of temperatures studied. They are defined as the temperature at which the contribution of ΔS and ΔH to the free-energy change (ΔG) becomes 0. At the point of intersection of the two lines in the ΔG versus $T\Delta S$ plot (Figure 4A), the entropic contribution to the binding process becomes 0 at $\Delta G = \Delta H = -33.18$ kJ/mol. This intersection point corresponds to a temperature, T_s, at which the entropic contribution to the Gibbs energy of binding becomes favorable to unfavorable (*23*). From the linear fit of Figure 4A, T_s corresponding to $\Delta H = -33.18$ kJ/mol came at a temperature of 33.5 °C. Above this temperature, the reaction is enthalpy-driven. An enthalpic component of T_s exists namely, T_H, which is the temperature at which the enthalpic contribution to the Gibbs free energy changes from unfavorable to favorable (*24*). The temperature (T_H), corresponding to a 0 value of ΔH calculated from Figure 3B is approximately 21 °C. Below T_H, the binding process is completely entropy-driven, whereas at temperatures above T_s, the binding process is completely enthalpy-driven. In the intermediate range between temperatures of 21−33.5 °C, the reaction is influenced by both enthalpic and entropic factors. The behavior described above is often seen for other systems that are also accompanied by a large negative ΔC_p value (e.g., the thermodynamics of the transfer of benzene from aqueous solution to pure liquid) (*25*). The negative ΔS value at higher temperatures (33−37 °C), as shown in Table 1, is unfavorable for the podophyllotoxin−tubulin interaction. The ordering of water molecules at the complex interface contributes unfavorably to ΔS and favorably to ΔH (*26*). As such, the presence of additional factors counterbalancing the hydrophobic effect is indicated for the binding process at higher temperatures, which makes a larger contribution to the negative ΔS. These factors are (i) the constraint of intramolecular vibrational flexibility, (ii and iii) the reduction in the transnational and overall rotational degrees of freedom, and (iv) the conformational freezing of some amino acids in the protein because of drug binding (*27*). The empirical method of Sturtevant was used to estimate the hydrophobic and intramolecular vibrational contribution to ΔS (*28*), and the values are tabulated in Table 3. From Table 3, it is evident that the value of $\Delta S_{vibration}$ decreases along with temperature, making ΔS negative at higher temperatures. This is due to the restricted mobility of the drug upon complex formation with tubulin, resulting in the constraint of intramolecular vibrational flexibility, which then increases gradually at higher temperatures. The value of ΔC_p, which is grossly negative, is again an outcome of a hydrophobic ($\Delta C_{p_{hydrophobic}}$) as well as vibrational contribution ($\Delta C_{p_{vibrational}}$), resulting from respective heat-capacity-change factors (*29*).

ΔC_p Value for the Podophyllotoxin−Tubulin Interaction: Theoretically versus Experimentally Determined Values. There are numerous instances where the heat capacity changes upon protein−protein interactions or protein unfolding and can therefore be calculated on the basis of the changes in the water accessible surface area (ASA) for a given process (*30*−*32*). This requires knowledge of the three-dimensional structure of the corresponding complex. The crystal structure of the podophyllotoxin−tubulin complex is known from Protein Data Bank (PDB ID 1SA1) (*33*). Thus, the surface area for the free protein was calculated on the basis of this complex after removing the coordinates for the ligand. The change in ASA upon complex formation is estimated as follows:

$$\Delta ASA = ASA_{tubulin-podophyllotoxin} - ASA_{tubulin} - ASA_{podophyllotoxin} \quad (2)$$

where $ASA_{tubulin-podophyllotoxin}$ is the ASA of the complex, $ASA_{tubulin}$ is the ASA for the protein in the absence of podophyllotoxin, and $ASA_{podophyllotoxin}$ is the ASA for podophyllotoxin. The ASA values were calculated using the NACCESS computer program [designed by S. J. Hubbard and J. M. Thornton (*34*)] and are presented in Table 4. The ΔASA values are used to calculate the expected ΔC_p upon complex formation

$$\Delta C_p = 2.14\Delta ASA_{alp} + 1.55\Delta ASA_{arm} - 1.81\Delta ASA_{bb} - 0.88\Delta ASA_{pol} \quad (3)$$

where, the subscripts alp, arm, bb, and pol represent the change in ASA for aliphatic, aromatic, backbone, and polar atoms, respectively (*35*). On the basis of these ASA values, calculated according to the eq 2, the calculated ΔC_p is equal to −2.094 kJ mol^{-1} K^{-1}. This value is in proximity with the experimentally obtained one (−2.47 kJ mol^{-1} K^{-1}). There are instances of good agreement between the calculated and

Table 4: ASA of the Drug−Tubulin Complex

protein−drug complex	area of all atoms (Å^2)	area of nonpolar side chain (Å^2)	area of polar side chain (Å^2)	area of backbone (Å^2)	area of total side chain (Å^2)	resultant buried area (Å^2)
$tubulin_{colchicine}$[a]	31 279.9	17 485.4	9122.7	4610.2	26 608.1	
tubulin−colchicine complex	31 009.5	17 212.6	9104.7	4571.7	26 317.3	891.4
$tubulin_{podophyllotoxin}$[a]	32 429.6	17 897	9586.2	4909.2	27 483.1	
tubulin−podophyllotoxin complex	32 160.3	17 664	9570.8	4875.4	27 234.8	858.2
tubulin−AC complex	31 074.3	17 276	9117.2	4586.1	26 393.2	700
colchicine	621					
podophyllotoxin	588.9					
AC	494.7					

[a] Upon complex formation, colchicine is known to cause a conformational change in tubulin. Therefore, during the surface-area calculations, we considered the final form of the altered tubulin structure, which is visible in the crystal structure. Areas of $tubulin_{colchicine}$ and $tubulin_{podophyllotoxin}$ are separately used for the calculation for each respective drug−protein complex area calculation.

experimentally obtained ΔC_p values (*36*, *37*), which are indicative of interactions following the lock-and-key-binding model. The weaker complexes have a tendency to show greater ΔC_p values, owing to their enhanced enthalpic and entropic fluctuations at a less tight complex interface (*38*).

The active participation of hydrophobic forces or the role of entropy factors in the podophyllotoxin−tubulin binding process corroborates satisfactorily with the change in accessible surface area (ΔASA) values upon the drug−protein complex formation (Table 4). It is evident from Table 4 that the ΔASA upon complex formation is more or less uniform (slightly lower for the podophyllotoxin−tubulin interaction) for both podophyllotoxin−tubulin and colchicine−tubulin complex formation. In comparison, the AC−tubulin interaction is an enthalpy-driven reaction (Table 2) and exhibits a comparatively lower ΔASA value (ΔASA = 700 Å^2) with respect to the ΔASA values for the podophyllotoxin−tubulin and colchicine−tubulin complexes. Importantly, the smaller ΔASA value for AC compared to podophyllotoxin indicates that the podophyllotoxin−tubulin interaction involves not only ring A but also participation from additional rings (B, C, D, and/or E) of the drug skeleton. This conclusion is also supported by previous studies involving modifications in the D ring of podophyllotoxin, which sharply indicate the presence of at least one additional point of attachment for podophyllotoxin with tubulin (*3*). Therefore, the connection of podophyllotoxin to tubulin encompasses a large area and excludes water from the drug−protein interfaces. A considerable portion of podophyllotoxin is embedded within the *β*-tubulin structure as viewed from the crystal structure (Figure 5). This close association of the drug with tubulin would explain its high negative ΔC_p value.

Binding Studies with Podophyllotoxin Analogues with Altered Oxalone and Lactone Rings. From the crystal structure (PDB ID 1SA1) of the tubulin−podophyllotoxin complex, it was observed that a threonine residue (Thr_{179}) of the α subunit of tubulin is present in close proximity with the 4′-hydroxy group of ring D of podophyllotoxin (Figure 5). The measured distance (approximately 3.4 Å) ensures a strong possibility of the interaction between the carbonyl oxygen of the peptide bond of the threonine residue on α-tubulin with the 4′-hydroxy group of ring D on podophyllotoxin. To confirm the involvement of the oxalone moiety as well as the lactone ring (ring E) of podophyllotoxin in tubulin binding, we synthesized three analogues of podophyllotoxin, whereby the hydroxy substitution at the 4′ position of ring D is replaced by a methoxy group (analogue I) along with its isomer (analogue II) and another analogue

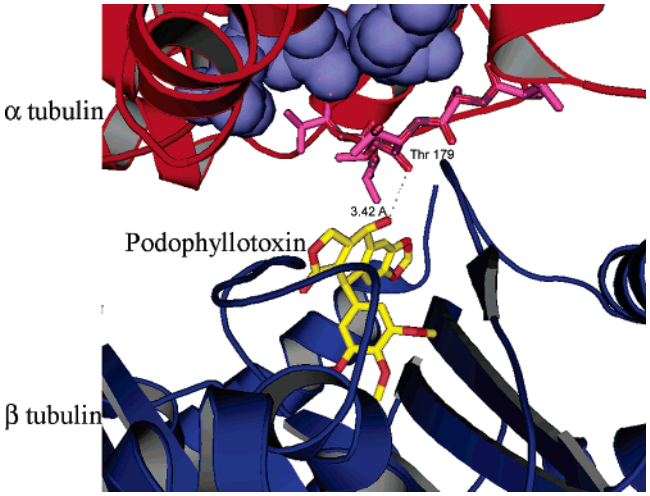

FIGURE 5: Crystal structure of the tubulin−podophyllotoxin complex. A close overview showing the possibility of hydrogen bonding between a threonine residue (179) of tubulin with the 4′-hydroxy group on ring D of podophyllotoxin. A considerable portion of the tetralin moiety of the drug is deeply embedded in the *β*-tubulin protein core.

that is epimerized at ring E (analogue III). With regards to various podophyllotoxin analogues, some cytotoxic data exist; however, the in vitro inhibition of tubulin polymerization was not determined for these podophyllotoxin analogues (*11*). Therefore, we measured the extent of inhibition of tubulin polymerization by our different podophyllotoxin analogues. The 4′-methoxy derivative of podophyllotoxin (analogue I) was effective at inhibiting tubulin polymerization at a concentration of 10 μM by 25% (IC_{50} values were not determined), whereas analogues II and III were ineffective up to 200 and 600 μM concentrations (data not shown).

To evaluate the exact nature of the interaction (i.e. whether hydrogen-bonding or noncovalent van der Waals interactions), we tested the binding of the 4′-methoxy derivatives of podophyllotoxin with tubulin. With these analogues, the probability of hydrogen bonding has been eliminated because of the unavailability of hydrogen, which has been replaced by a methyl group. We also studied the binding of the 4′-methoxy derivative of podophyllotoxin (analogue I) with tubulin using the aid of ITC (Figure 6A). The thermodynamics of binding along with the affinity constant are tabulated in Table 5. The binding thermodynamics of analogue I is very similar to that of podophyllotoxin (Table 1). It binds tubulin with an enthalpy value of $\Delta H = -12.44$ kJ/mol and an entropy of $\Delta S = 61.15$ J mol^{-1} K^{-1}. This binding rules out the necessity of hydrogen bonding between the hydroxy

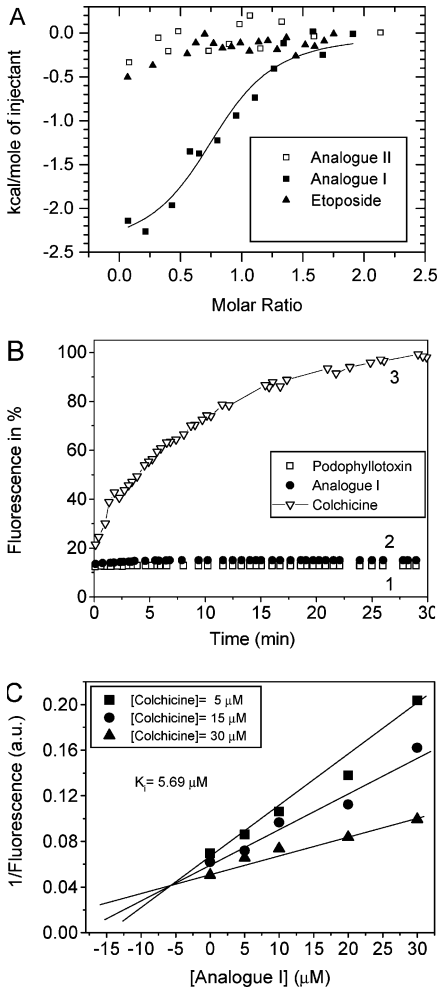

FIGURE 6: (A) Comparative view of calorimetric binding of podophyllotoxin analogue I (■), analogue II (□), and etoposide (▲). (B) Fluorimetric binding of podophyllotoxin along with its 4′-methoxy analogue I to tubulin with time. Podophyllotoxin and analogue I (50 μM) were incubated with tubulin (5 μM) for 15 min at 37 °C. The complex was then mixed with colchicine (50 μM), and the fluorescence value of colchicine was monitored at 430 nm up to 30 min. Podophyllotoxin (□), analogue I (●), and colchicine (▽). (C) Modified Dixon plot. The concentrations of colchicine were 5 μM (■), 15 μM (●), and 30 μM (▲). The reaction mixture contained tubulin (5 μM) and 4′-methoxy podophyllotoxin analogue I at the indicated concentration, and they were incubated at 37 °C for 45 min.

Table 5: Thermodynamics of Tubulin−Podophyllotoxin Analogue I Binding

temperature (K)	ΔG (kJ/mol)	ΔH (J/mol)	ΔS (J mol^{-1} K^{-1})
298	−30.58	−12.44	61.15

group of ring D and Thr_{179} in the podophyllotoxin−tubulin interaction. Thus, the nature of the interaction between podophyllotoxin and tubulin may potentially be related to the van der Waals type. To explore the effect of the stereochemical orientation of the methoxy group at the 4′ position on ring D of podophyllotoxin, we tested the binding of podophyllotoxin analogue II with tubulin. Surprisingly, no binding was observed (Figure 6A), which is in agreement with previous literature, whereby marked differences in the biological activity of cis−trans isomers in the D ring of podophyllotoxin were observed (*3*). We also studied the binding of podophyllotoxin analogue III (also known as the picropodophyllotoxin derivative), which was epimerized in ring E with tubulin. Notably, this compound showed insignificant binding with tubulin in ITC. This indicates that a proper orientation of the drug, not only in the substituent of ring D but also in other parts of the drug (like ring E), is crucial for effective binding. Finally, etoposide (where the hydroxyl group is replaced by a sugar group) also showed negative binding data with tubulin when analyzed with ITC (Figure 6A). Thus, the bulky substituent present on ring D, as in the case of etoposide, abolishes the binding ability of the compound completely. This result also provides strong evidence in support of oxalone and lactone moieties of podophyllotoxin in tubulin binding.

To determine whether podophyllotoxin analogue I binding with tubulin occurs in a reversible mode or not, podophyllotoxin and analogue I (50 μM) were incubated with tubulin (5 μM) for 15 min at 37 °C in a different set. The complex was then mixed with colchicine (50 μM). The fluorescence value of colchicine was monitored at 430 nm up to 30 min. It was observed from Figure 6B that podophyllotoxin and analogue I bind irreversibly to tubulin (curves 1 and 2).

To determine whether podophyllotoxin analogue I binding occurs at the colchicine-binding site, it was allowed to compete with colchicine for binding to tubulin and the data were analyzed using a modified Dixon plot (Figure 6C). The drugs were incubated with tubulin for 45 min at 37 °C. The results presented in Figure 6C clearly indicate that binding of analogue I was inhibited competitively by colchicine, yielding an apparent K_i value of about 5.69 μM. Etoposide (a nonbinder of tubulin), however, does not inhibit colchicine binding (data not shown).

DISCUSSION

Podophyllotoxin competes for the colchicine-binding site on tubulin. This podophyllotoxin-mediated competitive inhibition of colchicine binding has been ascribed to the fact that both compounds possess a trimethoxy phenyl ring (A ring). Nevertheless, there are differences in the binding properties of the two ligands. Unlike the colchicine−tubulin interaction, which is followed by a two-step reaction mechanism, podophyllotoxin−tubulin binding occurs in a single step. The podophyllotoxin−tubulin association reaction is faster and is accompanied by a relatively low energy of activation (61.74 kJ/mol) compared to that of colchicine (84−92.4 kJ/mol) (*3*). In addition, when compared with colchicine, no structural changes occur upon podophyllotoxin binding to tubulin as is seen in the case of colchicine binding (*39*).

Podophyllotoxin and colchicine also differ significantly with respect to GTPase activity. The presence of GTPase activity exhibited by colchicine is markedly absent for podophyllotoxin (*12*). This type of feature is not only unique for podophyllotoxin but is also observed for drugs such as RPR112378 and RPR115781. Similar to podophyllotoxin, these compounds bind at the colchicine site on tubulin without stimulation of GTPase activity (*40*). Podophyllotoxin binds tubulin through its A ring, showing initial stimulation of GTPase activity; however, it later causes inhibition because of its tetralin moiety, thereby indicating the participation of this portion of the drug in tubulin association (*12*). Previously, there have been reports that 4′-dimethyl deoxypodophyllotoxin-β-D-glucoside, which possesses a bulky

substituent in the 4′ position of podophyllotoxin ring A, is ineffective in inhibiting either colchicine or podophyllotoxin binding. This demonstrates that the availability of ring A is critical for podophyllotoxin−tubulin binding (*3*). Involvement of the rest of the ring portion of podophyllotoxin is apparent from other reports, such as the succinylation of the OH group of ring D on podophyllotoxin. This modification abolished the binding of the drug to tubulin (*41*). In addition, podophyllotoxin derivatives such as etopside (containing a sugar residue attached on the same hydroxy group of ring D) are devoid of antimicrotubular properties and instead inhibit DNA topoisomerase II binding (*1*).

The thermodynamics of podophyllotoxin−tubulin binding has revealed an entropy-dominated reaction with an unfavorable enthalpy (*3*). The thermodynamics of colchicine−tubulin interactions was studied in different laboratories, while in most cases, thermodynamic parameters were calculated from vant Hoff's plot (*3*). Menendez et al. first determined those parameters using microcalorimetry (*42*). Although there is consensus among entropy values among different groups, there is disagreement regarding enthalpy values of the colchicine−tubulin interaction. However, the use of ITC for the determination of thermodynamic parameters provides for more reliable results compared to those determined from vant Hoff's plot.

In this study, the thermodynamics of the podophyllotoxin tubulin interaction was studied across a range of temperatures. We observed a favorable enthalpy change at higher temperatures that is partly offset by an unfavorable entropy change in the calorimetric study. The large ΔC_p value ($\Delta C_p = -2.47$ kJ mol^{-1} K^{-1}) of the podophyllotoxin−tubulin interaction confirms the establishment of a hydrophobic contact between the drug and protein interfaces. Andreu and Timasheff have also suggested the predominance of a hydrophobic interaction in podophyllotoxin−tubulin binding (*5*). In addition, the enthalpically driven podophyllotoxin−tubulin reaction suggests the presence of a hydrogen-bonding interaction in the binding process. These two seemingly contrasting observations for podophyllotoxin−tubulin are not uncommon, because in many antigen−antibody reactions, the simultaneous participation of hydrogen-bonding and hydrophobic interactions has previously been noted (*43*−*46*).

The active participation of hydrophobic forces or the role of an entropy factor in podophyllotoxin−tubulin binding also corroborates satisfactorily with ΔASA values upon drug−protein complex formation (Table 4).

Recently, a report on different colchicine site binding drugs strongly suggested the presence of a common pharmachophore for the tubulin−drug interaction (*47*). The study obtained information through molecular modeling and suggested that for better binding a number of drug attachment points to the binding site were necessary. For podophyllotoxin, they identified six attachment points (encompassing the hydroxy substituent on ring D as well as some portions of the four rings B, C, D, and E), whereas for colchicine, the number of attachment points was five. In our studies with the podophyllotoxin analogues, we showed experimentally that this pharmacophoric proposition corroborates well up to a certain extent. The nature and stereochemistry of the 4′-hydroxy group on ring D of podophyllotoxin as well as ring E are crucial components, with most modifications yielding several congeners that have emerged as promising drug candidates.

The use of podophyllotoxin since ancient times as a clinical medicine has been restricted because of its toxicity. Despite this fact, podophyllotoxin still provides a basic key structure in structure−activity relationship (SAR) studies. As a result, several derivatives of podophyllotoxin have been synthesized and clinically tested (*48*). Among these derivatives, etoposide and teniposide have been frequently used in the clinical treatment of small cell lung cancer, testicular carcinoma, lymphoma, and Kaposis sarcoma because they inhibit DNA topoisomerase II activity and induce apoptosis (*48*). Etopside, however, is ineffective in inhibiting tubulin polymerization. It seems surprising that the same drug skeleton with modifications at a specific site (4′-hydroxy group of ring D of podophyllotoxin) would provide for a different mechanistic property. Therefore, in the work presented herein, we have studied the thermodynamics of the podophyllotoxin−tubulin interaction and analyzed the role of various fundamental forces involved in the binding process. This study on the thermodynamic mapping of the binding site of podophyllotoxin delineates differences in podophyllotoxin binding to tubulin compared to colchicine binding, despite the fact that they both bind at the same site on tubulin. This information may provide some insight into the development of future generation podophyllotoxin drugs.

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