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A mechanistic study of the binding of TN-16 to tubulin

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3-(1-Anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16), a synthetic compound, has previously been reported to have anti tumor effect and to inhibit microtubule assembly *in vitro*. In this study, we found that TN-16 inhibits microtubule assembly efficiently with an IC_{50} value of 3 mM. The low IC_{50} is interesting from the point of view of cancer treatment. Though TN-16 recognizes colchicine site on tubulin its binding is more like 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC) in terms of reversibility, high on rate, low activation energy and pH independency. Its binding thermodynamics indicates that its binding occurs with a ΔCp value of –75.92 cal/mol/K that is also close to ΔCp value of –69 cal/mol/K for AC.

Keywords: Tubulin, TN-16, calorimetry, fluorescence, pH effect.

Introduction

Microtubules are important cytoskeletal components whose polymerization-depolymerization status or subtle changes in dynamics has serious implications in mitosis, intracellular transport and cellular motility[1–4]. Tubulin targeting agents like paclitaxel, vinblastine, vincristine, estramustine have wide spread application in the frontline treatment of cancer chemotherapy although colchicine cannot be used successfully as an antimitotic drug[5–8]. Both toxicity and drug resistance are major challenges for successful anticancer treatment as observed in the case of colchicine[9]. Nguyen *et al*. identified pharmacophoric attachment points for a number of colchicine site binding agents (CSI)[10]. Some of them bear similarity in structure with colchicine having trimethoxy phenyl ring (A ring) common with colchicine along with some structurally dissimilar drugs. Among the second group of colchicine site binding agents (CSIs), Indanocine. E-7010 and methoxyestradiol are potential candidates[11–13]. They are promising candidates because E-7010 and methoxy estradiol are now in clinical trial[14,15]. Indanocine also exhibits effectivity against multidrug resistant cell lines[11]. With increasing number of drugs becoming ineffective for cancer treatment, the search for potential anticancer drugs is never over[16,17]. TN-16 has previously been reported to have anti tumor effect[18] and to inhibit microtubule assembly *in vitro*[19]. It is structurally different from colchicine. TN-16 inhibits colchicine binding to tubulin. About 3 μM TN-16 was required for 50% inhibition of the taxol-induced assembly of tubulin[19]. TN-16 also has been shown to arrest cells in metaphase and hence, used in synchronizing cells to stay at mitotic stage[20]. TN-16, being a microtubule assembly inhibitor, is a suitable candidate for further research. Kinetics as well as thermodynamic study has not been carried out with this compound so far.

So our primary objective with this drug was to perform kinetic along with thermodynamic study of TN-16 binding to tubulin. Additionally, as it has little structural resemblance with colchicine, we also want to check whether it behaves like (AC) 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone or colchicine during binding to tubulin. 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (AC) being simple in structure than colchicine binds fast to tubulin in a pH independent manner[21–23]. It has low energy of activation and the interaction is enthalpy driven[24]. On the contrary, the binding of colchicine to tubulin is slow, pH dependent, nearly irreversible, has high energy of activation and entropy driven[24–26]. Our data suggests binding nature of TN-16 analogous with AC rather than with colchicine when binding to tubulin is concerned.

Materials and methods

Tubulin isolation and estimation:

Microtubular proteins were isolated from goat brains by

two cycles of a temperature-dependent assembly-disassembly process. Pure tubulin was isolated from microtubular proteins by two additional cycles of temperature-dependent polymerization and depolymerization using 1 *M* glutamate buffer for assembly[27]. The composition of the assembly buffer was 50 mM PIPES, pH 6.9, 1 mM EGTA, 0.5 mM $MgCl_2$ and 0.5 mM GTP. The protein was stored at –70°C. The protein concentration was determined by the method of Lowry *et al.*[28] using bovine serum albumin as standard.

Binding measurements by fluorescence method:

The binding of the ligands to the protein was monitored by enhancement of ligand fluorescence in the presence of protein. Fluorescence spectra were recorded using a Hitachi F-3000 fluorescence spectrophotometer connected to a constant temperature circulating water bath accurate to ±0.2°C. All fluorescence measurements were carried out in a 0.5 cm path-length quartz cuvette, and fluorescence values were corrected for the inner filter effect using the following equation of Lakowicz[29]:

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

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

To investigate whether TN-16 binds tubulin reversibility or not, the binding spectra were recorded in the fluorescence spectrometer. Initially 5 μM tubulin was mixed with 5 μM of TN-16 for 15 min at 37°C. After saturation, excess amount (60 μM) of colchicine was added to the reaction mixture and time dependence binding was monitored fluorimetrically. For control reaction, same experiment was performed without TN-16. Excitation and emission wavelengths used for the measurement were 353 and 430 nm and the excitation and emission band pass were 10 and 5 nm respectively, in all cases.

Modified dixon plots of TN-16 was obtained using colchicine as a competitive inhibitor. The reaction mixtures containing tubulin (3 μM), different concentrations of colchicine (0–20 μM) and TN-16 (0–30 μM) were incubated at 37°C for 60 min. The reciprocal of the fluorescence intensity of the podophyllotoxin-tubulin complex at 430 nm was plotted against the concentration of TN-16. The resulting Dixon plot gave an approximate K_i value for TN-16 of 7.5 μM (Supplementary Fig. 1S).

Association rate and activation energy:

The association kinetics of TN-16 (10 μM) with tubulin (1 μM), were measured under pseudo-first order conditions (where the drug was present in a large excess over tubulin) using a Hitachi F-3000 spectrofluorometer. The ligand was added to the tubulin solution and the quenching of tryptophan emission at 336 nm was measured on excitation at 280 nm (slit widths of 5 nm). All fluorescence measurements were carried out in a 0.5 cm path length quartz cuvette. A_{280}, A_{336} are the absorbance at the excitation and emission wavelength respectively. The biphasic plot was analysed according to Lambier and Engelborghs[30]:

$$F_{Qmax} - F_{Qt} = A.e^{-\alpha t} + B.e^{-\beta t}$$

where F_{Qmax} is the maximum intensity of quenched fluorescence, F_{Qt} is the quenched fluorescence at time *t*, *A* and *B* are the amplitudes and α, β are the rate constants for the fast and slow phases respectively. The amplitude of the slow phase *B* was low relative to the fast phase *A*, and the slow phase was not analyzed further[31]. The apparent on-rate constants (k_{on}) were calculated as $k_{on} = \alpha/c$ where *a* is the slope in the semilogarithmic plot ln (F_{Qmax}–F_{Qt}) versus time (*t*) and *c* is the concentration of drug. This data analysis was done using software Microcal Origin 5.0. The association rate constant k_{on} was determined at different temperatures ranging from 22 to 37°C and activation energy (E_a) was calculated by plotting k_{on} against (1/*T*) according to the Arrhenius equation, $k_{on} = A \exp(-E_a/RT)$, where *A* is the pre-exponential factor. The temperature was controlled with a circulating water bath (NesLab) and was accurate to 0.2°C.

Calorimetry:

Isothermal titration calorimetric measurements were performed on a VP-ITC MicroCalorimeter of MicroCal, Inc. (Northampton, MA). Tubulin (30 μM was dialyzed extensively against PEM buffer with 0.1 mM GDP (to offer stabilization), and the TN-16 was 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 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.

Results

In vitro binding of TN-16 to tubulin TN-16 binds tubulin in a reversible manner:

It was known from the earlier studies that TN-16 inhibits colchicine binding to tubulin[19]. We tried to verify it again. In this experiment, TN-16 was allowed to compete with colchicine for binding to tubulin and the results were analyzed using a modified Dixon plot[32]. It was observed that TN-16 inhibited colchicine binding with an approximate K_i value of 7.5 μM (Supplementary Fig. 1S). (Experimental details have been represented under Materials and method section). TN-16 in spite of having dissimilar structure from that of colchicine recognizes and binds to the same site. At this point we are inquisitive to know whether its binding to tubulin is reversible or not. It has no intrinsic fluorescence and does not induce fluorescence upon binding to tubulin. So the extent of reversibility of the TN-16-tubulin interactions was quantitated by chasing the preformed drug-tubulin complex with an excess of colchicine and monitoring the increase in the colchicine-induced fluorescence. Tubulin was preincubated for 30 min at 37°C with TN-16, and was transferred to a cuvette placed in a fluorimeter maintained at 37°C with a temperature-controlled water bath and chased with an excess of colchicine. As a result, we detected the formation of the fluorescent colchicine-tubulin complex following the dissociation of tubulin from the TN-16-tubulin complex. The binding profile clearly indicates that TN-16 binds to tubulin in a reversible way while the colchicine binding to tubulin is mostly irreversible[25] (Fig. 2). The extent of formation of the colchicine-tubulin complex was taken as a measure of reversibility of the TN-16-tubulin interactions.

Association rate and the activation energy:

Association rate and the activation energy of colchicine and its analogues binding with tubulin are related to the B ring and the side chain at C-7 position[24]. For colchicine-tubulin binding the on-rate and activation energy are 130 M^{-1} s^{-1} at 37°C and 20 kcal/mol respectively. On the other hand, AC binds to tubulin with comparatively lower activation en-

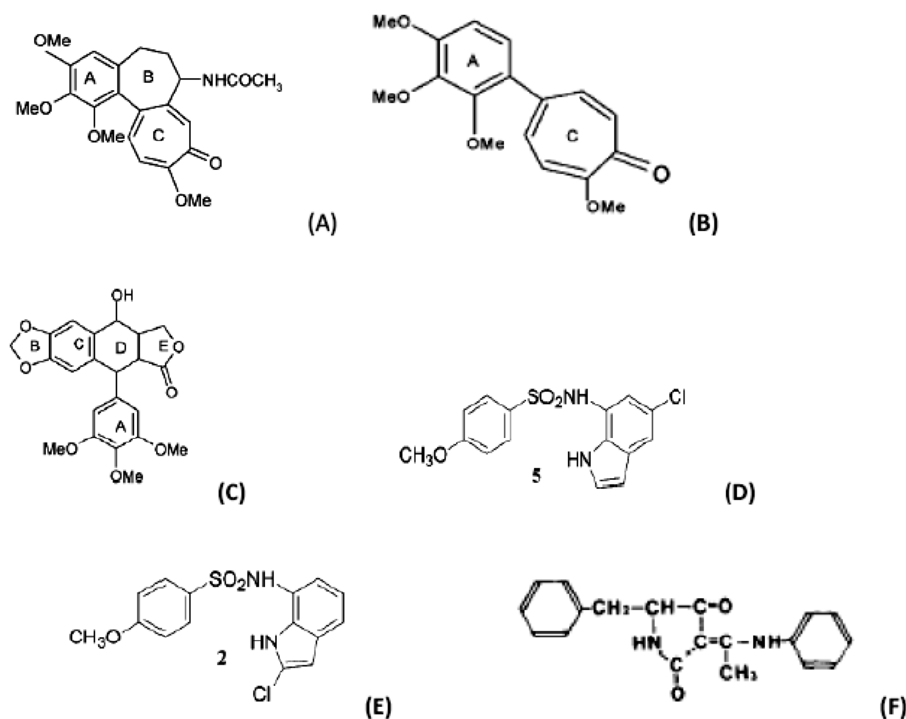

Fig. 1. Structure of drugs: (A) Colchicine, (B) AC, (C) Podophyllotoxin, (D) Sulfonamide drug 5, (E) Sulfonamide drug 2, (F) TN-16.

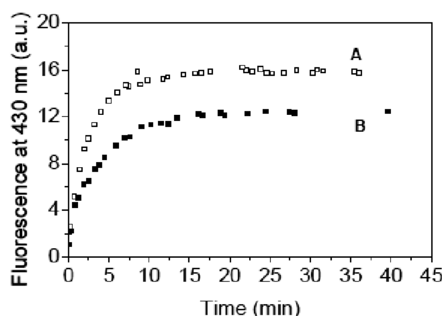

Fig. 2. Reversibility of binding of TN-16 to tubulin, (A) Binding of 5 μM tubulin and 60 μM colchicines (□); (B) Preformed complex of 5 μM tubulin and 5 μM TN-16, chased with 60 μM colchicines ( ).

ergy with respect to colchicine-tubulin binding. Interaction of AC with tubulin is followed from the enhancement of AC fluorescence upon binding tubulin. The association process can be resolved into a fast and slow phase similar to that of colchicine. The apparent second order rate constant for the fast phase is 5.2×10^4 $M^{-1}s^{-1}$ at 37°C and the activation energy is 13 kcal/mol. It was supposed that AC-tubulin binding occurs through a low energy pathway due to the uninterrupted free rotation about the biaryl bond. Similar to AC, the association rate and the activation energy has been calculated for the TN-16-tubulin binding. As it has no intrinsic fluorescence and do not induce fluorescence upon binding to tubulin, so its binding to tubulin has been monitored through the quenching of tryptophan fluorescence. Association rate and activation energy for TN-16-tubulin binding is 1264.54 M^{-1} s^{-1} at 31°C and 8.12 kcal/mol respectively (Fig. 3). High on rate and very low activation energy of TN-16-tubulin binding is reminiscent of AC tubulin binding.

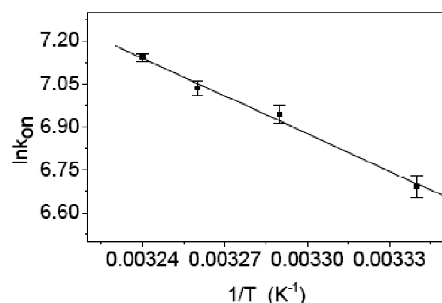

Fig. 3. Effect of temperature on the association rate constant of TN-16 to tubulin. Details of the experiment were given in the 'Materials and methods' section.

TN-16 binds tubulin in a pH independent manner unlike colchicine:

The interaction of colchicine with tubulin is strongly influenced by the pH of the binding reaction whereas the binding of a colchicine analogue lacking B ring (such as AC) with tubulin is little influenced by the pH (Fig. 4). At this stage we want to check the pH dependency of binding for TN-16 with tubulin at different pH values. Since it is known that TN-16 fluoresce poorly upon binding to tubulin, we studied it's binding using quenching of the tryptophan fluorescence of tubulin[31]. Fig. 4 shows that the extent of TN-16 binding to tubulin is not largely different at the all pH studied.

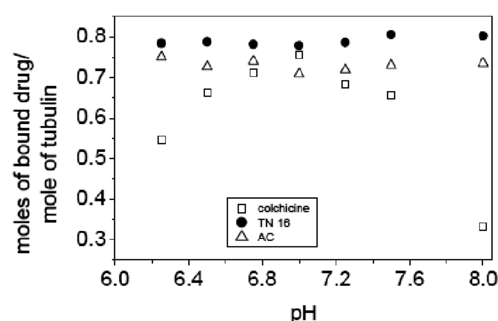

Fig. 4. Effect of pH: Binding of colchicine (□), AC (Δ) and TN-16 (•) to tubulin at various pHs as measured by fluorescence. Tubulin and drug concentrations were 2 μM and 30 μM in case of colchicine and AC. Complexes were excited at 350 nm, and emission was measured at 430. For TN-16, the complex was excited at 280 nm, and emission was measured at 336 nm.

Thermodynamics of TN-16-tubulin interactions:

The thermodynamics of TN-16-tubulin interaction was not done previously. Thermodynamic parameters such as Gibbs free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and heat capacity (ΔCp) can provide useful information to identify fundamental forces involved in protein-drug interaction. So to decipher the nature of interaction of TN-16 with tubulin, the thermodynamics of binding of TN-16 with tubulin was performed using ITC. Fig. 5 shows the raw data of a calorimetric experiment, which involved the titration of tubulin with TN-16 in PEM buffer at 30°C. The thermodynamic parameters ΔH and ΔS are determined over a range of temperatures from 25 to 37°C and are presented in Table 1. Fig. 5 reveals the enthalpy change (ΔH) upon binding as a function of the concentration of TN-16 for each injection.

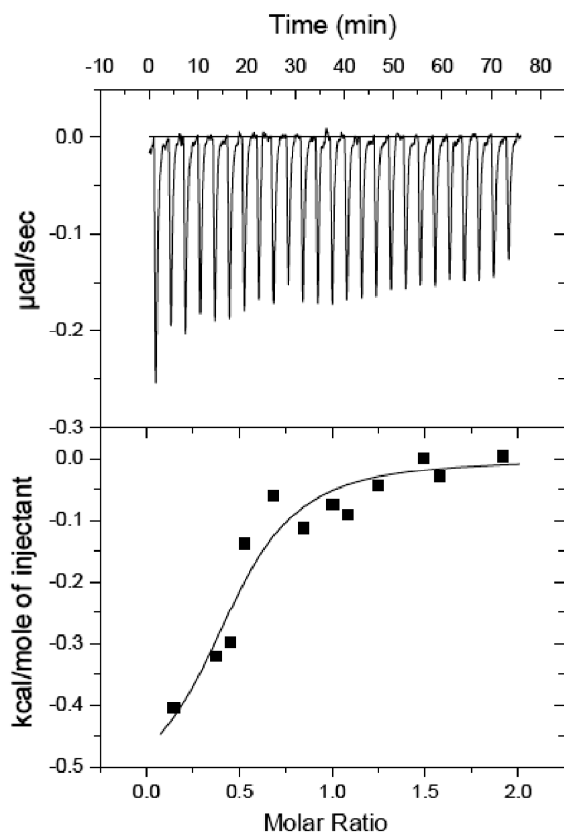

Fig. 5. Calorimetric titration of tubulin with TN-16 (Raw data obtained from 25 injections of 10 µL aliquots of TN-16 to 0.025 µM tubulin in 50 mM PIPES buffer pH 7.0). (B) Nonlinear least-squares fit of the incremental heat per mole added TN-16 for the titration in A by the injection number as a function of the molar ratio using origin.

Table 1. Thermodynamics of tubulin-TN-16 binding

Temperature (K)	ΔH (cal/mol)	ΔS (cal/mol/K)	ΔCp (cal/mol/K)
298	–723.5	21.6	–75.92
302	–1067	18.94	
307	–1497	18.64	
310	–1607	19.07	

The heat capacity change at constant pressure (ΔCp) is determined using Kirchoff's equation as:

$$d\Delta H/dT = \Delta Cp \quad (1)$$

A plot of enthalpy change (ΔH) of TN-16-tubulin binding as a function of temperature yields ΔCp = –75.92 cal/mol/K (Fig. 6). It is evident from earlier experimental results that the reaction of AC with tubulin is enthalpy driven along with a much

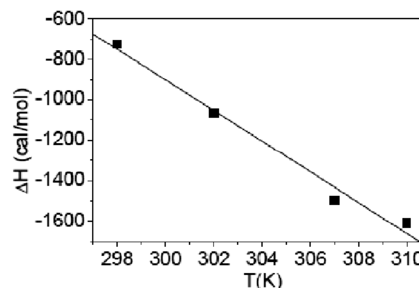

Fig. 6. Temperature dependence of the enthalpy change (ΔH) upon binding of TN-16 to tubulin at pH 7.0. The continuous line is the least-squares fit of the data.

less negative ΔCp value (–69 cal/mol/K)[32]. Another valuable parameter emerging out from ITC experiment is the change in entropy factor (ΔS). It consists of three terms and can be presented as follows)[33]:

$$\Delta S = \Delta S_{solv} + \Delta S_{conf} + \Delta S_{mix} \quad (2)$$

Using this relationship it is possible to determine the extent of conformational changes induced by ligand binding. Once ΔCp has been determined, the entropy change at a reference temperature (T_r) can be calculated using eq. (3).

$$\Delta S = \Delta S_{TR} + \int_{T_R}^{T} \Delta Cp d \ln T \quad (3)$$

The reference temperature normally corresponds to temperature at which hydration is zero. Baker and Murphy have proposed that the entropy of both polar and apolar solvation is close to zero near 385 K[33]. Number of experimental results also certifies this temperature to be 385 K. For entropy change in case of transfer of six liquid hydrocarbons (This can be considered as a model for hydrophobic interaction in protein folding that comes with a zero value at temperature 385.5±2.2 K). Synonymous results have been noticed for eight different alcohols, apolar gases, saturated hydrophobic gases, and solid dipeptide after least square fit analysis of the data (Plotted entropy change against ΔCp)[34,35].

So using T_R = 385 and S_{TR} = 0

The eq. (3) can be rearranged to yield the following equation,

$$\Delta S_{solv,\ 298\ K} = \Delta Cp \ln (298\ K/385\ K)$$

The translational entropy (ΔS_{mix}) of the TN-16-tubulin bind-

Table 2. Division of the entropic term of TN-16-tubulin binding

Drug	ΔCp (cal/mol/K)	ΔS_{298} (cal/mol/K)	ΔS_{mix} (cal/mol/K)	$\Delta S_{solvation}$ (cal/mol/K)	$\Delta S_{conformation}$ (cal/mol/K)
TN-16	–75.92	21.6	–7.98	19.2	10.4
AC[a]	–69	5.93	–7.98	17.67	–3.75
Podophyllotoxin[a]	–589	17.67	–7.98	151	–125.35
Sulfonamide[b] (Drug 2)	–589	8.7	–7.98	150.9	–134.22
Sulfonamide[b] (Drug 5)	264	19.2	–7.98	–67.63	94.81

[a,b]Values used from studies by Gupta *et al.* 2006 and Banerjee *et al.* 2005.

ing reaction can be calculated using equation,

$$\Delta S_{mix} = R \ln (1/55.5)$$

ΔS_{mix} is a measure of entropy change due to the mixing of TN-16 and solvent molecule that is generated as a result of entropy change due to the changes in translational and rotational degrees of freedom.

The availability of ΔCp value for binding of TN-16 to tubulin enables us to estimate the ΔS_{solv} using eq. (3). Table 2 represents the value corresponding to ΔS_{solv} and ΔS_{conf} for TN-16-tubulin binding. ΔS_{conf} is positive (10.4 cal/mol/K) thus playing the main role in order to drive the binding of TN-16 to tubulin.

Discussion

Colchicine binds to tubulin in a slow nearly irreversible manner upon long incubation at 37°C for equilibration. On the other hand, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropone (AC) binds tubulin instantaneously in a reversible way[22,23]. TN-16 binds tubulin in a reversible way like AC.

TN-16 binds tubulin in a pH independent manner unlike colchicine:

At present, the three-dimensional structure of the tubulin dimer is known[36] and the colchicine binding site is identified at the α/β interface with the C-terminal α-tail too far from the colchicine binding site to have a direct interaction with bound colchicine. Earlier studies reveal that the neutralization of negative charges of α-C-terminus of tubulin either by subtilisin digestion or in presence of salt leads to major changes in colchicine-tubulin interaction[26]; namely, the reaction becomes pH-independent and has lower activation energy. However, no significant changes in pH are observed for colchicine analogues lacking B-ring like AC. Here TN-16 behaves more like AC or like Tubulin S ($\alpha_s\beta_s$) as far as the effect of pH is considered[26].

Thermodynamics of TN-16-tubulin binding:

With the knowledge that binding of TN-16 to tubulin occurs in a reversible manner at the colchicine binding site, we want to compare its binding in light of thermodynamics. Binding of TN-16 to tubulin occurs with a positive ΔS_{conf} (+10.4 cal/K/mol) with a positive ΔS_{solv} of 19.2 cal/K/mol. When similar parameterizations of entropy factors has been done for AC-tubulin binding (change in entropy value taken from previous ITC data of AC-tubulin binding[32], it was accompanied with ΔS_{conf} value of –3.75 cal/K/mol with relatively high positive value of ΔS_{solv} of 17.67 cal/K/mol. Since calorimetric studies for colchicine-tubulin binding cannot be done due to very slow binding of colchicine, in this case we consider podophyllotoxin, another colchicine site binding (CSI) agent having trimethoxy phenyl ring (ring A) common to colchicine. The main structural difference of this compound from that of AC is its relatively big structure with a tetrahydronaphthol ring along with A ring of colchicine. The reason behind that this compound has been selected here for discussion is its irreversible binding nature like colchicine. It also shares common features like pH dependency while binding to tubulin though it has activation energy lower than colchicine by 3–4 kcal/mol. From its thermodynamics, it is known that this it's binding with tubulin is driven by enthalpy with an entropic penalty at higher temperature (31°K) with high negative ΔCp value (–589 cal/mol/K)[32]. When the value of ΔS_{conf} for podophyllotoxin-tubulin binding has been calculated (using thermodynamic data from our previous experiment), it is drastically negative with a ΔS_{conf} –125.35 cal/mol/K with ΔS_{solv} of 151 cal/mol/K. This binding is dominated by high negative ΔS_{conf} in contrast to TN-16-tubulin and AC-tubulin binding.

As ΔS_{conf} decreases from +10.4 cal/mol/K to –125.35 cal/mol/K from TN-16 to podophyllotoxin, it indicates different extents of conformational changes suffered during binding of these drugs to the colchicine site. This decrease in the ΔS_{conf} is expected due to the bigger structure of podophyllotoxin in comparison to AC and TN-16, its binding will occur with greater surface association with tubulin. High negative ΔCp value (–589 cal/mol/K) also corresponds to this association. Podophyllotoxin expels more water upon its binding from the site of association on tubulin than relatively flexible, shorter in structure TN-16 and AC. Hence it looses more conformational freedom upon complexation. Being smaller in size TN-16 binds tubulin easily with a much lower energy of activation. AC also follows the same path with a little bit negative ΔS_{conf} value (–3.75 cal/mol/K). On the other hand sulfonamides (Drug 2 and 5) with minimum structural difference among them (Fig. 1D, 1E) presented positive as well as negative ΔS_{conf} values[37]. It changes from –134.22 to 94.81 cal/mol/K from drug 2 to 5. The difference in the position of chlorine substituent creates differences in ΔS_{conf} values for sulfonamides. It has also been reflected from their ΔCp values that changes from –589 to 264 cal/mol/K. Much lower ΔCp value observed in TN-16-tubulin binding indicates lesser extent of surface-surface association between protein-drug interfaces. Among all the thermodynamic parameters enthalpy, entropy, and free energy can originate from a variety of sources but change of heat capacity due to binding provides information about solvation only[38–42]. From this thermodynamic study one thing is very clear that in spite of recognizing the same binding site, different drugs bind tubulin in their own fashion with different thermodynamic parameters contributing differently to the binding. TN-16 binding to tubulin is more akin to AC rather than podophyllotoxin and sulfonamide (Drug 2) binding taken for comparison instead of colchicine.

Conclusions

TN-16-tubulin binding has been studied and compared with colchicine-tubulin and AC-tubulin binding. TN-16 behaves in a more identical manner like AC rather than colchicine. It binds tubulin in a fast, reversible way with high association rate and low activation energy. It also inhibits tubulin polymerization efficiently with an IC_{50} value of 3 μM (figure not shown). It also binds tubulin in a pH independent manner similar to AC. Not only that its binding thermodynamics corresponds to AC with respect to ΔCp value. So it can be interpreted that though TN-16 recognizes the colchicine-binding site on tubulin and binds there but its binding profile is entirely different from that of colchicine. It resembles AC more likely and acts as a simple structurally dissimilar AC analogue.

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