Sulfonamide Drugs Binding to the Colchicine Site of Tubulin: Thermodynamic Analysis of the Drug−Tubulin Interactions by Isothermal Titration Calorimetry

Mithu Banerjee,[†] Asim Poddar,[†] Gopa Mitra,[†] Avadhesha Surolia,[‡] Takashi Owa,*[,§] and Bhabatarak Bhattacharyya*[,†]

Department of Biochemistry, Centenary Campus, P1/12, CIT Scheme 7 M, Bose Institute, Calcutta 700 054, India, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India, and Laboratory of Seeds Finding Technology, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan

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The discovery of several sulfonamide drugs paved the way toward the synthesis of **6** (*N*-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide, E7010) and **7** (*N*-(3-fluoro-4-methoxyphenyl)pentafluorobenzenesulfonamide, T138067), both of which inhibit tubulin polymerization and are under clinical development. A series of diarylsulfonamides containing an indole scaffold was also found to have antimitotic properties, but their mode of interactions with tubulin has remained unidentified so far. In this study, we demonstrate that these sulfonamide drugs bind to the colchicine site of tubulin in a reversible manner. They quenched intrinsic tryptophan fluorescence of tubulin presumably due to drug-induced conformational changes in the protein, but were unable to modulate GTPase activity of tubulin in contrast to colchicine that enhances the same enzymatic activity. Further investigation using isothermal titration calorimetry (ITC) revealed that **5** (*N*-(5-chloro-7-indolyl)-4-methoxybenzenesulfonamide) afforded a large positive value of heat capacity change ($\Delta C_p = +264$ cal mol^{-1} K^{-1}) on binding to tubulin, suggesting a substantial conformational transition in the protein along with partial enthalpy−entropy compensation. On the other hand, the 2-chloro regioisomer **2** gave a large negative value of ΔC_p (−589 cal mol^{-1} K^{-1}) along with complete enthalpy−entropy compensation. This thermodynamic profile was thought to be attributable to a prominent contribution of van der Waals interaction and hydrogen bonding between specific groups in the drug−tubulin complex. These results indicate that a mere alteration in the position of a single substituent chlorine on the indole scaffold has a great influence on the drug−tubulin binding thermodynamics.

Introduction

Looking back at the long history of drug discovery, one will find that a specific group of drugs having a common functional unit often exhibits diverse biological activities. The sulfonamides define such a group, well-known for a variety of pharmacological effects such as antibiotic, hypoglycemic, diuretic, and antihypertensive activities.[1] This serendipitous finding has stimulated research efforts aiming at the discovery of a novel class of antitumor sulfonamides.[2] One example of this class of small molecules is drug **6**, which was discovered from sulfonamide-focused compound libraries (Figure 1B).[3,4] Interestingly, the potential of **6** to inhibit microtubule assembly by binding to tubulin at the colchicine site was noted.[5] Because of its good antitumor activity in vivo against both rodent tumors and human tumor xenografts, **6** has entered clinical trials as an orally active tubulin polymerization inhibitor.[6] Another example to be mentioned is drug **7**, which was shown to prevent microtubule formation by its selective and covalent modification of tubulin at the Cys-239 residue in the colchicine binding site (Figure 1B).[7] Notable in vivo antitumor effects against multidrug resistant (MDR) subline xenografts have promoted this compound to clinical evaluation.

Tubulin is a major molecular target for antitumor drugs that disrupt cellular mitosis.[8] Tubulin-targeting agents are generally divided into two classes: (i) microtubule-stabilizing agents such as taxanes, epothilones, and discodermolide; and (ii) microtubule-destabilizing agents such as colchicine, vinca alkaloids, and cryptophycins.[9,10] While taxanes and vinca alkaloids are widely used clinical drugs for the treatment of cancer, it should also be noted that colchicine-related small molecules, including **6**, **7**, combretastatins, and their analogues, have recently attracted a great deal of attention because of their promising in vivo antitumor efficacy accompanied by antivascular action (Figure 1B).[11−13] In this situation, a new generation of antitumor sulfonamides based on the structure of **6** has been designed.[4,14] This generation of sulfonamide drugs contains an indole scaffold in place of the amino-substituted pyridine ring of **6**. As shown in Figure 1A, sulfonamide drugs **2**−**5** differ in the position of a single substituent chlorine, while drug **1** bears no such substituent. Drugs **2**−**5** have been named according to the position of the substituent. All of them were shown to inhibit tubulin polymerization in low μM concentrations and to exhibit potent antiproliferative activity against a number of cancer cell lines.[4,14] Although an ap-

* To whom correspondence should be addressed. B.B.: phone, +91-33-2337-9544; fax, +91-33-2334-3886; e-mail, bablu@boseinst.ernet.in. T.O.: phone, +81-29-847-7442; fax, +81-29-847-7614; e-mail, t-owa@hhc.eisai.co.jp.
† Bose Institute.
‡ Indian Institute of Science.
§ Eisai Co., Ltd.

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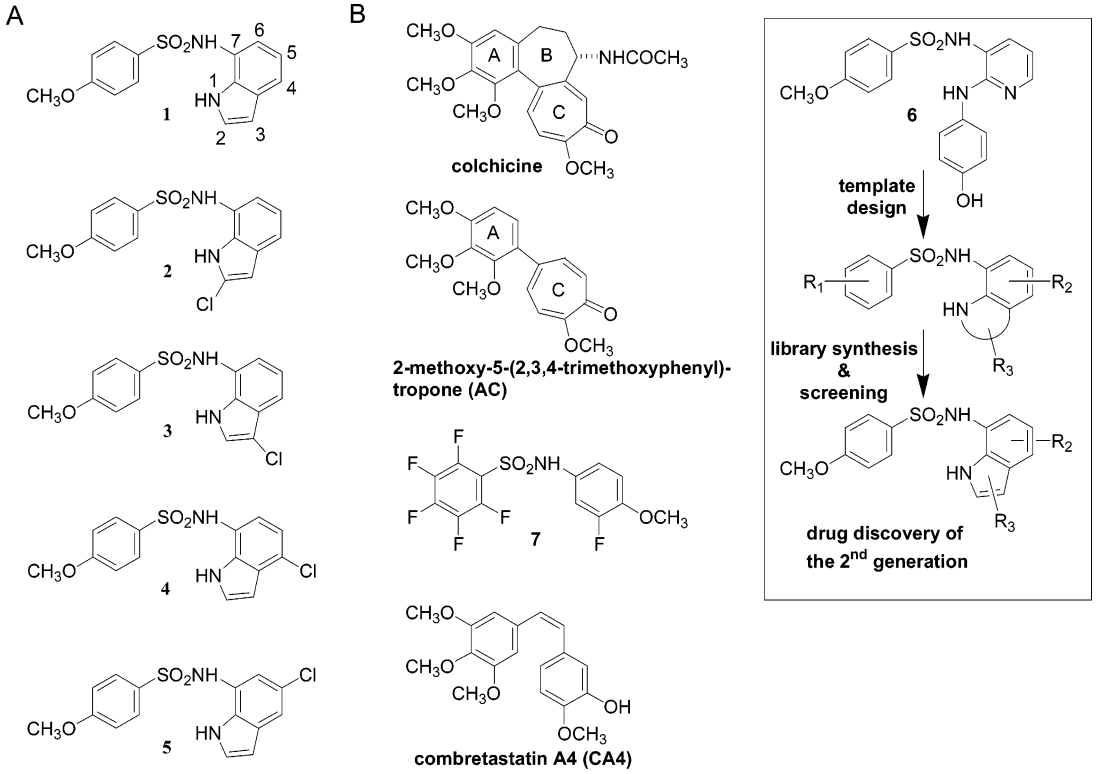

Figure 1. Structures of various antimicrotubule drugs. (A) Antimitotic sulfonamides with the indole scaffold. (B) Structures of some antimitotic drugs resembling colchicine and drug discovery flowchart of the second generation sulfonamides (shown in the box).

preciable advancement in the potency of these drugs was confirmed at cellular levels, their binding properties with tubulin have remained unidentified.

This paper for the first time explores the mode of interactions of these promising sulfonamide drugs with tubulin. One of the most important attempts herein is to investigate the influence of a minor alteration in the position of the chloro substituent on the binding properties. For this research purpose, we used isothermal titration calorimetry (ITC) analysis with particular focus on the drug−tubulin binding thermodynamics. Our study represents a small molecule based approach to better understanding drug−tubulin interactions and may also be useful for designing improved colchicine-related antitubulin chemotherapeutics.

Results

Drug Binding Site and Reversibility of the Binding. It is generally observed that drugs inhibiting tubulin polymerization interact with tubulin either at the vinca site or at the colchicine site. It was reported that the sulfonamide drug **6** binds tubulin rapidly and competes with colchicine for binding.[5] Sulfonamide drugs **1**−**5** shown in Figure 1A are structurally different from **6** as they contain the indole scaffold instead of the pyridine ring of **6**. Although these sulfonamide drugs resemble colchicine possessing a 2,3,4-trimethoxyphenyl group only in having a 4-methoxyphenyl group, the rest of their structures are entirely different from that of colchicine (Figure 1A,B). As reported previously all these compounds are capable of inhibiting tubulin polymerization in low μM concentrations, thereby exhibiting antimitotic and antiproliferative activities in cell-based assays with an approximate rank order of **2** $\approx$ **5** > **1** $\approx$ **3** $\approx$ **4**.[4,14] We therefore tried to examine whether this second generation of sulfonamide drugs inhibit colchicine binding to tubulin. In our assay, drugs **1**−**5** were allowed to compete with colchicine for binding to tubulin and the results were analyzed using a modified Dixon plot (data not shown). Consequently, colchicine binding was inhibited competitively by all these sulfonamide drugs with estimated K_i values of 14.0, 28.0, 14.0, 14.0, and 7.0 μM for drugs **1**, **2**, **3**, **4**, and **5**, respectively. (The procedure used for this measurement is described in the Experimental Section.)

Colchicine binding to tubulin is a slow process, which needs long incubation at 37 °C for equilibration. On the other hand, colchicine analogues devoid of the B ring such as 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone (AC, Figure 1B) bind tubulin instantaneously.[15,16] Colchicine−tubulin interaction is poorly reversible, while AC binding to tubulin is reversible. Like AC, these sulfonamide drugs are structurally flexible in nature as rotation about the sulfonamide bond is possible. We therefore wanted to investigate whether drugs **1**−**5** bind tubulin reversibly. They have no intrinsic fluorescence and do not induce fluorescence upon binding to tubulin. Hence, the extent of reversibility of the drug−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 each sulfonamide drug to be tested (at three times the concentration of each K_i value), and then this drug−tubulin complex 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 (at twice that of the sulfonamide

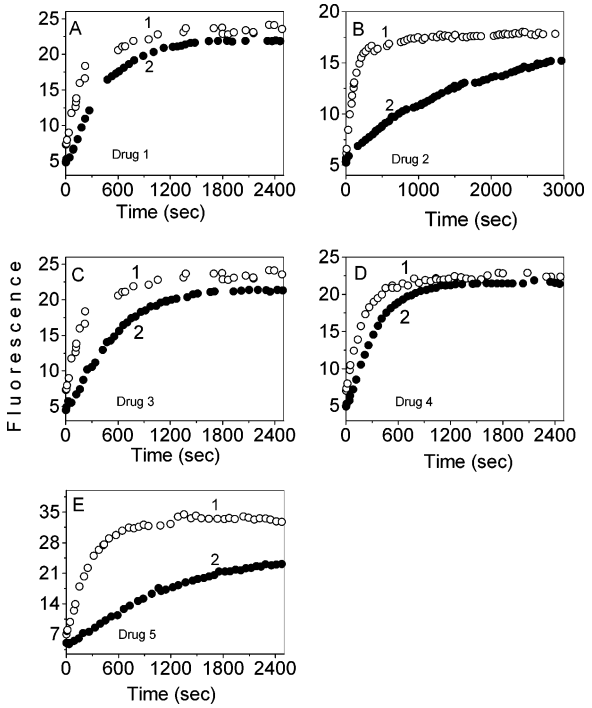

Figure 2. Reversibility of binding of sulfonamide drugs to tubulin. (A) Curve 1, binding of 4 μM tubulin and 84 μM colchicine; curve 2, preformed complex of 4 μM tubulin and 42 μM drug **1**, chased with 84 μM colchicine. (B) Curve 1, binding of 4 μM tubulin and 168 μM colchicine; curve 2, preformed complex of 4 μM tubulin and 84 μM drug **2**, chased with 168 μM colchicine. (C) Curve 1, binding of 4 μM tubulin and 84 μM colchicine; curve 2, preformed complex of 4 μM tubulin and 42 μM drug **3**, chased with 84 μM colchicine. (D) Curve 1, binding of 4 μM tubulin and 84 μM colchicine; curve 2, preformed complex of 4 μM tubulin and 42 μM drug **4**, chased with 84 μM colchicine. (E) Curve 1, binding of 4 μM tubulin and 42 μM colchicine; curve 2, preformed complex of 4 μM tubulin and 21 μM drug **5**, chased with 42 μM colchicine. After repeated experiments, the representative data plots for all test drugs were selected as shown here.

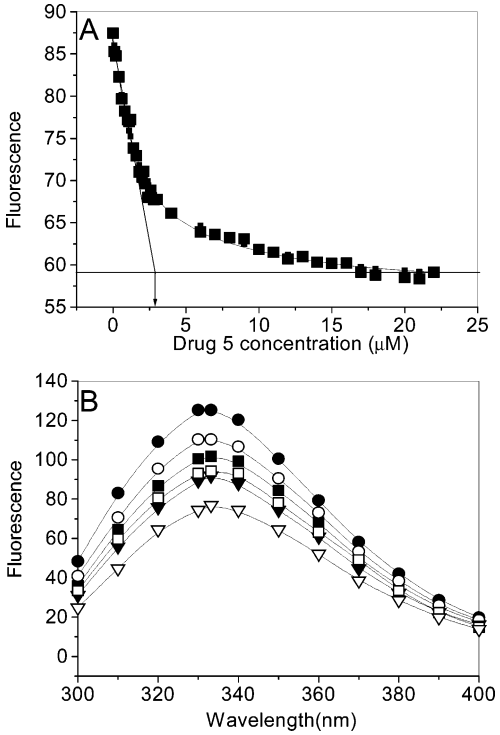

Figure 3. Quenching of the tryptophan fluorescence of tubulin by sulfonamide drugs. (A) Dissociation constant of tubulin−drug **5** complex was determined by using quenching of the tryptophan fluorescence of tubulin. Tubulin concentration was fixed at 1 μM, and titration was performed by gradual addition of drug **5** at 25 °C. The abscissa value at the intersection between the tangent to the initial portion of the curve and the asymptotic line is equal to $n[T_t] + K_d$, where $[T_t]$ and n are the initial concentration and the number of the binding site(s) of tubulin, respectively. Here we got an abscissa value of 3.0 μM. The value of $n = 1$ was taken from the calorimetric experiment. The reciprocal of $K_d = 2.0$ μM gave $K_a = 0.5$ μM^{-1}, which correlates well with the binding constant obtained from the calorimetric data. (B) Tryptophan fluorescence emission spectra of tubulin (1 μM) in the absence (●) and in the presence of drug **1** (■), drug **2** (○), drug **3** (▼), drug **4** (□), and drug **5** (▽). Drug concentration was 20 μM in all cases. Standard errors for all data points are in the range of ±10% of the plotted values.

drug concentration in each). As a result, we detected the formation of the fluorescent colchicine−tubulin complex following the dissociation of tubulin from each sulfonamide drug. This clearly indicates that in all these sulfonamide drugs the binding to tubulin is reversible while the colchicine binding to tubulin is mostly irreversible. The extent of formation of the colchicine−tubulin complex was taken as a measure of reversibility of the sulfonamide drug−tubulin interactions. As shown in Figure 2, the extent of reversibility appears to be greater for drugs **1**, **3**, and **4** than drugs **2** and **5**.

Drug-Induced Conformational Changes in Tubulin. Colchicine binding to tubulin involves conformational changes in both tubulin and colchicine. It is well-known that colchicine stimulates GTPase activity of tubulin. This enhanced enzymatic activity of tubulin is considered a consequence of drug-induced conformational changes in tubulin. Although colchicine enhances the GTPase activity, AC has no such activity. We observed that unlike colchicine these sulfonamide drugs could neither enhance nor suppress the GTPase activity of tubulin, suggesting that they fail to induce colchicine-like dramatic conformational changes in tubulin. (The raw data in this assay are not shown here, but the procedure is available in the Experimental Section.) However, local changes in the conformation of tubulin, especially at the binding site, seem to occur as is evident from changes in tryptophan fluorescence upon their binding to tubulin (Figure 3B). Chymotrypsin cleaves β-tubulin only at Tyr-281, producing fragments β-N and β-C. According to Sackett and Verma,[17] colchicine-bound tubulin has an additional chymotrypsin sensitive site at or near the C-terminus of α-tubulin. Thus, enzymatic digestion was carried out for 3 min at 30 °C with the chymotrypsin-to-tubulin ratio of 1:100. (The procedure used for this assay is described in the Experimental Section.) In consequence, these sulfonamide drugs produced no additional band (data not shown). This suggests that these drugs, despite their binding at or near the colchicine site, were unable to induce colchicine-like conformational changes in tubulin.

Tubulin contains eight tryptophan residues, and thus an alternative method to look at drug-induced conformational changes in tubulin is to monitor the tryptophan fluorescence of tubulin in the presence of these sulfonamide drugs. In fact, colchicine-induced confor-

mational changes in tubulin were established by chasing the attenuation of this tryptophan fluorescence emission. We therefore tested effects of these sulfonamide drugs on the tryptophan fluorescence of tubulin, leading to the observation of quenching of the fluorescence emission by all drugs. The concentration-dependent change in the tryptophan fluorescence of tubulin by drug **5** and quenching of the fluorescence emission by each of five sulfonamide drugs are shown in Figure 3A and Figure 3B, respectively. In the titration experiment, the final fluorescence value was corrected for any possible inner filter effect. All the sulfonamide drugs quenched the tryptophan fluorescence to a different extent, drug **5** being the best quencher. A possibility of quenching through the energy transfer mechanism fails to provide an answer since these sulfonamide drugs possess no absorption in the emission region of tryptophan. Therefore, a subtle alteration in the conformation of tubulin and/or the exposure of tryptophan to the solvent may explain the observed quenching. An analysis of the quenching data[18,19] yielded the binding constant for drug **5** (Figure 3A), which correlated well with the binding constant obtained from the ITC measurement. This indicates that the drug **5** induced change in fluorescence intensity truly reflects events associated with its binding to tubulin.

Thermodynamics of Sulfonamide Drug−Tubulin Interactions. With recent advancement in the sensitivity and reliability of the calorimeter, ITC has become an important tool for the direct measurement of thermodynamic parameters in various biological interactions.[20,21] Moreover, ITC represents a universal detector that is dependent only on the exchange of heat during a reaction. Therefore, it provides an invaluable tool to monitor a variety of reactions independent of spectroscopic changes that occur during the reactions. ITC yields thermodynamic parameters such as Gibbs free energy change ($\Delta G_b°$), enthalpy change ($\Delta H_b°$), and entropy change (ΔS_b), along with the number of binding sites (N) and affinity constant (K_a) in a single experiment. Also determination of binding enthalpy as a function of temperature yields changes in heat capacity associated with an interaction that provides a valuable insight into the type and magnitude of forces involved therein. Therefore, we have utilized ITC to determine the thermodynamics of binding of these sulfonamide drugs to tubulin (Figure 4). Drugs **2**−**5** studied herein differ from each other in the position of a single substituent chlorine, and drug **1** bears no such substituent. We therefore tried to explore the influence of the position of the chloro substituent on the binding thermodynamics of the lead drug **1**.

Results of ITC studies on the interactions of these sulfonamide drugs with tubulin are summarized in Table 1. The data present a wealth of information on the mechanism of the binding of this novel class of drugs to tubulin. All the drugs exhibit the stoichiometry of one for their binding to tubulin. For drug **1**, lowering of the binding affinity occurs with increase in temperature, leading to a decrease in the free energy of binding. Since all thermodynamic parameters of drug **1** were highly temperature-dependent, there was no enthalpy−entropy compensation. The binding of drug **4** displayed favorable changes in enthalpy, whereas the binding of drug **3** was

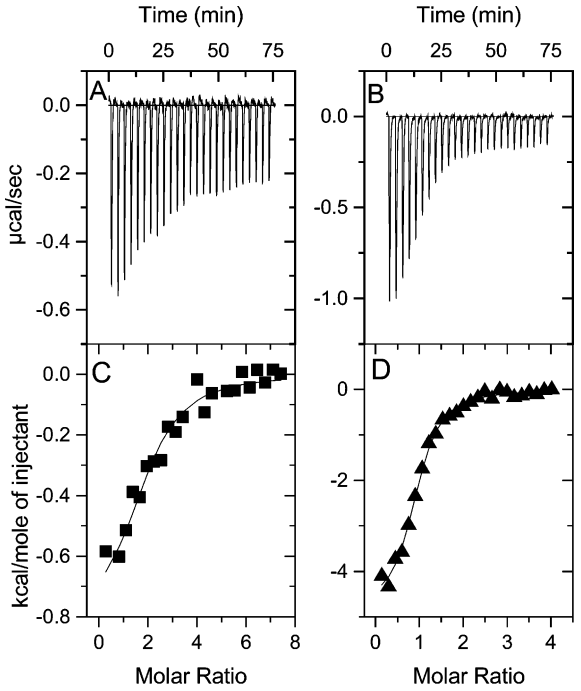

Figure 4. Calorimetric titration of tubulin with drug **1** and drug **2** at 25 °C. The upper panels A and B show raw data obtained from 25 injections (10 μL each) of drug **1** and drug **2**, respectively. The drug concentration in the syringe was 1500 μM in the case of drug **1** and 750 μM in the case of drug **2**, whereas the tubulin concentration was 38 μM for drug **1** and 35 μM for drug **2**. The lower panels C and D show plots of total energy exchanged (as kcal/mol of injectant) as a function of molar ratio of the ligand to the protein. Integrated curves are for drug **1** (■) and for drug **2** (▲).

favored entropically. Drugs **2** and **5** being more potent inhibitors of tubulin polymerization, the calorimetric titration for both drugs was carried out at different temperatures and the change in heat capacity (ΔC_p) associated with the binding reaction was determined. In the case of drugs **2** and **5**, both ΔH and ΔS showed clear temperature dependence. The free energy of binding did not vary greatly with temperature; moreover, ΔS varied in a compensatory manner with ΔH, more so in the case of drug **2** than drug **5**.

Enthalpy−entropy compensation is associated with solvent reorganization accompanying protein−ligand interactions.[22−26] A linear relationship of ΔH with $T\Delta S$ with slope exactly equal to one is an indication of complete compensation. Generally, enthalpy−entropy compensation occurs in any system with ΔC_p not equal to zero[27] and when $[\Delta C_p°] \gg [\Delta S°]$.[23,28,29] Figure 5A and Figure 5B show enthalpy−entropy compensation plots depicting the variation of ΔH as a function of $T\Delta S$ for drug **2** and for drug **5**, respectively. The slope that is $d\Delta H/d(T\Delta S)$ was equal to 1.04 for drug **2** and 0.625 for drug **5**. So we had complete enthalpy−entropy compensation for drug **2** and partial compensation for drug **5**. Such compensatory effect is well-known for protein−ligand binding.[30] Over a narrow temperature range, the temperature dependence of ΔH is given by $\Delta H = \Delta H° + \Delta C_p(T - T_0)$, where $\Delta H°$ is the binding enthalpy at an arbitrary reference temperature T_0 and ΔC_p is the heat capacity change of binding. The value of ΔC_p as determined from the slope of the linear fit in Figure 6A was +264 cal mol^{-1} K^{-1} for drug **5**, and it was −589 cal

Table 1. Thermodynamic Parameters of Sulfonamide Drug−Tubulin Interactions[a]

drug	temp °C (°K)	N	$K_a \times 10^{-5}$ M^{-1}	$-\Delta H$ kcal mol^{-1}	$-\Delta G$[b] kcal mol^{-1}	ΔS[c] cal mol^{-1} K^{-1}	ΔC_p[d] cal mol^{-1} K^{-1}
1	20 (293)	1.05 ± 0.03	6.60 ± 1.55	1.94 ± 0.08	7.74 (±0.15)	20.0 (±0.25)	+28
	25 (298)	1.07 ± 0.22	0.34 ± 0.12	1.80 ± 0.48	6.06 (±0.25)	14.70(±1.62)	
	30 (303)	1.08 ± 0.26	0.03 ± 0.01	1.66 ± 0.57	4.69 (±0.28)	10.00 (±1.89)	
2	20 (293)	1.30 ± 0.02	3.14 ± 0.27	3.12 ± 0.05	7.34 (±0.05)	14.50 (±0.16)	−589
	22 (295)	0.71 ± 0.02	2.93 ± 0.53	4.27 ± 0.23	7.33 (±0.11)	10.54 (±0.76)	
	25 (298)	0.90 ± 0.01	2.84 ± 0.20	4.85 ± 0.08	7.41 (±0.04)	8.70 (±0.24)	
	30 (303)	0.50 ± 0.01	2.94 ± 0.27	9.16 ± 0.28	7.55 (±0.06)	−5.21 (±0.65)	
3	25 (298)	1.30 ± 0.34	0.18 ± 0.07	0.87 ± 0.31	5.67 (±0.28)	16.60 (±1.03)	
4	25 (298)	0.95 ± 0.04	0.79 ± 0.11	15.63 ± 0.98	6.64 (±0.08)	−30.02 (±3.23)	
5	20 (293)	0.51 ± 0.04	1.13 ± 0.23	3.05 ± 0.33	6.73 (±0.12)	12.72 (±1.13)	+264
	22 (295)	1.30 ± 0.05	3.37 ± 1.06	3.02 ± 0.17	7.37 (±0.21)	15.07 (±0.56)	
	25 (298)	0.99 ± 0.02	4.36 ± 0.82	1.97 ± 0.08	7.64 (±0.12)	19.20 (±0.25)	
	27 (300)	0.98 ± 0.05	7.06 ± 3.01	1.31 ± 0.09	7.84 (±0.33)	22.39 (±0.31)	

[a] All values except for ΔC_p are means of three independent data ± SE. [b,c] ΔG and ΔS were calculated by the following equations, respectively: $\Delta G = -RT \ln K_a$; $\Delta S = (\Delta H - \Delta G)/T$. [d] ΔC_p (=dΔH/dT) was determined from the slope of the linear fit in Figure 6.

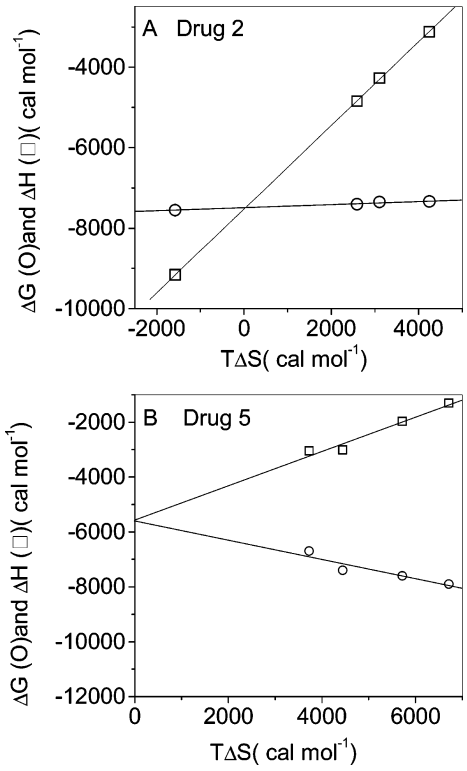

Figure 5. Enthalpy−entropy compensation plots for the binding of sulfonamide drugs to tubulin. (A) Dependence of ΔG (○) and ΔH (□) on $T\Delta S$ for drug **2**. (B) Dependence of ΔG (○) and ΔH (□) on $T\Delta S$ for drug **5**.

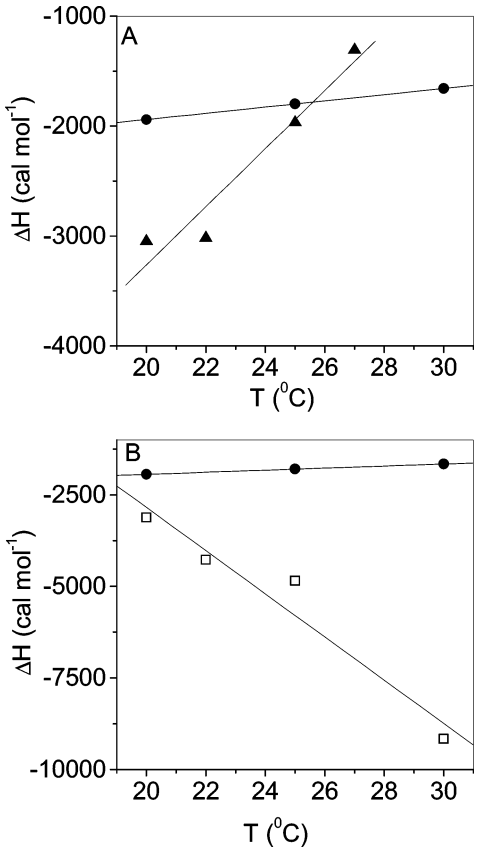

Figure 6. Enthalpy of the binding of sulfonamide drugs to tubulin as a function of temperature. (A) Drug **1** (●) and drug **5** (▲). (B) Drug **1** (●) and drug **2** (□).

mol^{-1} K^{-1} for drug **2** (Figure 6B). Thus, a minor alteration in the position of the chloro substituent brought about such a reversal of the sign of heat capacity.

Discussion

The observed clinical responses with drug **6**[31] and drug **7**[32] as well as the notable antivascular/antiangiogenic action of colchicine analogues[11,12] have recently stimulated research interest in small molecules binding to the colchicine site of tubulin. On the basis of this background, we decided to examine the second generation of antimitotic sulfonamides **1**−**5**, with particular focus on the mode and thermodynamics of their interactions with tubulin. While conventional cell-based assays could not discriminate clearly between drug **2** and drug **5** in terms of their antimitotic and antiproliferative potencies, our ITC analysis has revealed a remarkable difference in their binding thermodynamics. In fact, drug **5** gives a large positive value of ΔC_p (+264 cal mol^{-1} K^{-1}) along with partial enthalpy−entropy compensation whereas drug **2** gives a large negative value of ΔC_p (−589 cal mol^{-1} K^{-1}) along with complete enthalpy−entropy compensation.

The factors contributing to a positive value of ΔC_p are as follows: (a) solvent−drug interactions; (b) solvent interactions with amino acid side chains in the drug binding site; (c) changes in the drug conformation; and (d) changes in the protein structure as a result of the drug binding.[33] Both drugs **2** and **5** were dissolved in 100% DMSO, and the final DMSO concentration was 5% in the cell chamber during the binding reaction.

Since both drugs were under identical solvent conditions during the binding, the first possibility mentioned above is not a likely explanation. Solvent interactions with the amino acid residues within the drug binding site seem to be unlikely, as both of them bind to the same site and inhibit the colchicine binding to tubulin. Changes in the drug conformation as a result of the binding seem also very unlikely, as the basic structural framework is the same for both drugs. The only possibility left is changes in the protein structure as a result of the drug binding. Figure 6A reflects the temperature dependence of ΔH for drug **5**. ΔH and ΔS compensate each other, resulting in a close to linear dependence of ΔG on temperature (Figure 5B). Along with increase in temperature there is a decrease in ΔH and an increase in ΔS for drug **5**. It has been seen that drug **5** quenches tryptophan fluorescence to an appreciable extent, which may be due to some conformational change in tubulin. In fact, the increase of ΔS with increase in temperature was consistent with conformational changes in tubulin.[33] This conformational transition may contribute to the observed increase in ΔS due to increased protein−solvent interactions, thereby leading to the positive change in ΔC_p. Thus, in the case of drug **5** partial enthalpy−entropy compensation is also thought to result from the conformational transition in tubulin.

The binding of drug **2** to tubulin was mainly driven by favorable changes in ΔH. Thus, van der Waals interaction and hydrogen bonding might play a prominent role in the formation of the drug−tubulin complex as has been noted with several other protein−ligand interactions.[34] A large negative value of ΔC_p is usually taken as an indicator of a dominant hydrophobic effect related to the binding process. There is recent literature by Vulevic and Correia, which extensively reported on ΔC_p effects in the tubulin system.[35] According to this study, the majority of the effect comes from tubulin−tubulin interactions and involves the burying of nonpolar surface. A large negative heat capacity is most consistent with the burial of water-accessible hydrophobic surface area, which gives rise to the release of bound water. Also, it has been found that negative ΔC_p values for protein folding and protein−ligand associations[36] are proportional to the reduction in water-accessible nonpolar surface areas of the molecules and hence related to the contribution of hydrophobic effect to molecular association. The heat capacity change for drug **2** binding has a large negative value (−589 cal mol^{-1} K^{-1}), and we also have seen such a large negative ΔC_p value in the case of self-association or aggregation of protein. To examine this possibility in the thermodynamic analysis of drug **2**, we performed a dynamic light scattering experiment under the same conditions as used for the ITC experiment. In consequence, drug **2** did not induce self-association or aggregation of tubulin at all (data not shown). Thus the large negative value of ΔC_p was considered to result from drug **2** binding to tubulin. For interpreting our data quantitatively, the empirical method of Sturtevant[37] was used to estimate the hydrophobic and intramolecular vibrational contribution to ΔC_p for drug **2** (Table 2). As a result, the hydrophobic contribution to ΔC_p was found to be greater than the calculated vibrational contribution, corresponding to the prediction that the negative ΔC_p might originate from a favorable hydrophobic contribution.

Table 2. Hydrophobic and Vibrational Contribution to ΔC_p and ΔS for Drug **2**

temp °C	ΔC_p (hydro) cal mol^{-1} K^{-1}	ΔC_p (vib) cal mol^{-1} K^{-1}	ΔS (hydro) cal mol^{-1} K^{-1}	ΔS (vib) cal mol^{-1} K^{-1}
20	−483.17	−105.83	125.62	−111.12
22	−480.15	−108.85	124.84	−114.29
25	−478.74	−110.26	124.47	−115.77
30	−468.13	−120.87	121.71	−126.91

The hydrophobic effect generally brings two nonpolar surfaces closer together and depletes water from the binding surface, resulting in a positive change in ΔS. However, here we had the lowering of ΔS with an increase in temperature. Thus, there must be some other factors that overcome the hydrophobic effect, thereby making a greater negative contribution to ΔS. Again, the empirical method of Sturtevant[37] was used to estimate the hydrophobic and intramolecular vibrational contribution to ΔS (Table 2). For drug **2**, the sign of the calculated hydrophobic contribution to ΔS was positive whereas that of the calculated vibrational contribution to ΔS was negative. With an increase in temperature the vibrational contribution toward entropy increased but the hydrophobic contribution remained more or less constant, thereby leading to the lowering of ΔS.

Taken altogether, the enthalpy-driven binding of drug **2** to tubulin with an increase in temperature suggests that van der Waals interaction and hydrogen bonding between specific groups, together with increased hydrophobic contribution, gave rise to such a large negative value of ΔC_p. Although the decrease in ΔS may seem contradictory, it is not surprising as a tight and better binding would impose more restraint on the intramolecular vibrations of the complex.[34] The negative ΔC_p is thought to bring about the net thermodynamic driving force for the drug binding to shift from entropic to enthalpic with an increase in temperature. Such calculations could not be made for drug **5** since its binding to tubulin appeared to involve a lot of conformational changes in the protein and thus the assumption (conformational $\Delta S = 0$) required for the application of the empirical method of Sturtevant was not valid.

If the absolute value of ΔC_p is large enough, both ΔH and ΔS change in a highly temperature-dependent manner. In such a case the reversal of the sign of both ΔH and ΔS is not unusual, particularly over the temperature range of interest. By extension of Baldwin's terminology,[38] we defined T_H and T_s as the characteristic temperatures for the process of interest, where $\Delta H = 0$ and $\Delta S = 0$, respectively. The negative value of ΔC_p for drug **2** being large in nature, T_H and T_s were calculated for drug **2**. Figure 5A shows plots of ΔG and ΔH as a function of $T\Delta S$ for drug **2**. At the intersection point of both lines, $\Delta G = \Delta H = -7485$ cal mol^{-1} with the entropic contribution to the binding process being zero. This intersection point corresponds to the temperature T_s, at which the contribution of entropy to ΔG changes from favorable to unfavorable.[39] From the linear fit shown in Figure 6B, we had $\Delta H = -7485$ cal mol^{-1} connected with the T_s of 27.9 °C. We also found that above T_s (27.9 °C) there was change in the sign of ΔS from positive to negative (Table 1), indicating that the

reaction becomes entropically disfavored above 27.9 °C. The temperature T_H, at which the contribution of enthalpy to ΔG changes from unfavorable to favorable corresponding to the value of ΔH being zero, was estimated to be 15.2 °C from the linear fit of ΔH versus T (Figure 6B). Thus, at temperatures below T_H the binding of drug **2** to tubulin was favored by entropy, whereas at temperatures above T_s the binding process became enthalpy-driven. In the temperature range between T_s and T_H, that is from 27.9 °C to 15.2 °C, the drug binding to tubulin was favored both enthalpically and entropically (Table 1). Sturtevant observed that many noncovalent binding processes involving proteins exhibit standard heat capacity changes that are large in magnitude. Such a characteristic has been observed for binding processes accompanied by a large negative ΔC_p, such as the formation of specific protein−DNA complexes[38] and the binding of benzamidines to trypsin.[39]

Conclusion

In conclusion, our study has disclosed the mode of the interactions between tubulin and antimitotic drugs **1**−**5** that was discovered from sulfonamide-focused small molecule libraries. This sort of small molecule based research is currently recognized as a chemical biology approach to explore various biological systems.[40] Our results demonstrate that a minor alteration in the chemical structure, i.e. the position of a single substituent chlorine on the pharmacophore, exerts a significant influence on the drug−tubulin interactions. Of particular note is that the present ITC analysis has illuminated the distinctive difference between drug **2** and drug **5** in the binding thermodynamics even though previous cell-based assays were unable to distinguish their activity profiles. These data could be applicable for designing advanced antitubulin drugs with excellent antitumor properties. Further investigation should be warranted to clarify which of the two binding thermodynamic characters is more favorable for the in vivo antitumor efficacy and safety profile of this series of small molecules.

Experimental Section

General. All commercial solvents and reagents were used without further purification. Melting points were determined on a Yanagimoto micromelting point apparatus and are reported uncorrected. Proton (^{1}H) NMR spectra were obtained at 400 MHz on a Varian UNITY 400 spectrometer in DMSO-d_6. Chemical shifts are expressed in δ (ppm) units relative to tetramethylsilane (TMS) reference. Mass spectra (HRMS) were recorded on a JEOL JMS-SX102AAQQ using a direct introduction method in the EI+ ion mode. Analytical results indicated by element symbols were within ±0.4% of the theoretical values.

Materials. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), GTP, GDP, and $MgCl_2$ were purchased from Sigma. Malachite green and ammonium molybdate were bought from E. Merck and SRL, respectively. All other commercial reagents used were of analytical grade.

Test Drugs. Colchicine was purchased from Sigma, and AC compound (colchicine analogue) was a gift from T. J. Fitzgerald, Florida A&M University. The sulfonamide drugs **1**−**5** were prepared according to the solution-phase parallel synthesis as published previously.[4b] Drugs **1**, **2**, and **3** were characterized by spectroscopic and analytical data as described in the previous reports.[4a,14] All drugs being soluble in DMSO and their molar extinction coefficients being unknown, stock solutions at desired concentrations were prepared by dissolving meticulously weighed samples in calculated quantity of 100% DMSO. A maximum concentration of 5% DMSO was maintained under the assay conditions described below.

***N*-(4-Chloro-7-indolyl)-4-methoxybenzenesulfonamide (4).** Purification by high-performance liquid chromatography (eluted EtOAc−hexane) gave **4** as a white solid: melting point 170−171 °C (after recrystallization from EtOAc−hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (3H, s), 6.42 (1H, dd, J = 3.0, 2.2 Hz), 6.71 (1H, d, J = 8.0 Hz), 6.91 (1H, d, J = 8.0 Hz), 6.99−7.05 (2H, *AA*′BB′), 7.41 (1H, dd, J = 3.0, 2.2 Hz), 7.63−7.69 (2H, AA′*BB*′), 9.89 (1H, br s), 11.09 (1H, br s); HRMS (EI) M^+ calculated for $C_{15}H_{13}N_2O_3SCl$ 336.0337, found 336.0333. Anal. ($C_{15}H_{13}N_2O_3SCl$) C, H, N.

***N*-(5-Chloro-7-indolyl)-4-methoxybenzenesulfonamide (5).** Purification by high-performance liquid chromatography (eluted EtOAc−hexane) gave **5** as a yellowish white solid: melting point 144−145 °C (after recrystallization from EtOAc−hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (3H, s), 6.39 (1H, dd, J = 3.2, 2.0 Hz), 6.80 (1H, d, J = 1.6 Hz), 7.02−7.08 (2H, *AA*′BB′), 7.33 (1H, d, J = 1.6 Hz), 7.39 (1H, dd, J = 3.2, 2.0 Hz), 7.67−7.73 (2H, AA′*BB*′), 10.05 (1H, br s), 10.88 (1H, br s); HRMS (EI) M^+ calculated for $C_{15}H_{13}N_2O_3SCl$ 336.0337, found 336.0345. Anal. ($C_{15}H_{13}N_2O_3SCl$) C, H, N.

Tubulin Isolation. Microtubular proteins were isolated from goat brain by two cycles of temperature-dependent polymerization and depolymerization in PEM buffer containing 50 mM PIPES (pH 6.9), 1 mM EGTA, and 0.5 mM $MgCl_2$ in the presence of 0.5 mM GTP and 4 M glycerol. Pure tubulin free of microtubule-associated proteins was isolated from microtubular proteins by two more cycles of temperature-dependent polymerization and depolymerization using 1 M glutamate buffer for the assembly.[41] The protein was stored at −70 °C, and the concentration of tubulin was determined by the method of Lowry et al.[42] using bovine serum albumin as the standard.

Binding Assays. Modified Dixon plots of all these sulfonamide drugs were obtained using colchicine as a competitive inhibitor. The reaction mixtures containing tubulin (3 μM) and varied concentrations of colchicine (1.5−18 μM) and each of drugs **1**−**5** (0−40 μM) were incubated at 37 °C for 1 h. The reciprocal of the fluorescence intensity of the colchicine−tubulin complex at 435 nm was plotted against the concentration of each test drug. The resulting Dixon plots gave approximate K_i values for all sulfonamide drugs. For investigating the reversibility of binding of each sulfonamide drug to tubulin, the fluorescence spectra were recorded in a Hitachi F-3000 fluorescence spectrometer connected to a circulating water bath maintained at constant temperature. Excitation and emission wavelengths used for the measurement were 350 and 430 nm, respectively. The excitation and emission band-pass was 5 nm in all cases. Finally, quenching of the tryptophan fluorescence of tubulin by these sulfonamide drugs was monitored three times over to verify drug-induced conformational changes in tubulin. Intensities of the fluorescence emission at varied wavelengths between 300 and 400 nm were plotted with each sulfonamide drug. The affinity constant of drug **5** binding to tubulin was determined using the data plots of the tryptophan fluorescence at varied drug concentrations (0−22 μM). Excitation and emission wavelengths used for this assay were 280 and 335 nm, respectively. The excitation and emission band-pass was kept at 3 nm. The fluorescence was corrected for the inner filter effect using the following equation: $F_{corr} = F_{obs}\{\text{antilog}(A_{ex} + A_{em})/2\}$, where F_{corr} is the corrected fluorescence; F_{obs} is the observed fluorescence; and A_{ex} and A_{em} are optical densities at the excitation and emission wavelengths, respectively.

GTPase Assay. The effects of these sulfonamide drugs on tubulin-mediated hydrolysis of GTP were assessed using a malachite green colorimetric assay.[43,44] Tubulin samples (10 μM) were prepared in PEM (50 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 7.0) buffer with 100 μM sulfonamide drugs or colchicine. Samples were preincubated at 37 °C for 30 min. After the addition of GTP (0.1 mM), the reaction mixture was

divided into seven aliquots of 70 μL each and incubated at 37 °C. These aliquots were withdrawn at different time intervals, and the reaction was quenched with 7 μL of 7 M perchloric acid. Samples were centrifuged for 1 min using a microcentrifuge at full speed. From each sample 50 μL of the supernatant was added to 950 μL of a solution (containing 0.12% malachite green, 4.2% ammonium molybdate, and 0.3% Triton-X in 6 N sulfuric acid), mixed well, and kept in the dark for 1 h. Finally, the optical density was measured at 630 nm. The phosphate content of each sample was determined from the standard curve generated with known concentrations of inorganic phosphate.

Limited Protease Digestion. The limited protease digestion was carried out according to the method of Sackett and Varma.[17] Briefly, tubulin (10 μM) was incubated with or without colchicine and these five sulfonamide drugs separately for 30 min at 30 °C. Drug concentrations were 100 μM in the reaction mixture. Samples were digested for 3 min at 30 °C with chymotrypsin (0.01 mg/mL) by keeping the tubulin-to-enzyme ratio at 100:1. The reactions were stopped by adding 0.2 mM phenylmethanesulfonyl fluoride (PMSF). Then all samples were analyzed using SDS−PAGE (12% polyacrylamide gel) followed by Coomassie blue staining.[45]

Isothermal Titration Microcalorimetry (ITC). ITC measurements were performed on a VP-ITC calorimeter (Microcal Inc., Northampton, MA). Tubulin was dialyzed extensively against PEM buffer with 0.1 mM GDP, and the ligand (drug) was dissolved in the last dialysate. A typical titration involved 25 injections of each test drug (10 μL aliquot per injection) at 3-min intervals into the sample cell (volume 1.4359 mL) containing tubulin. The titration cell was stirred continuously at 310 rpm. The heat of the ligand dilution in the buffer alone was subtracted from the titration data for each drug. The data were analyzed to determine binding stoichiometry (N), affinity constant (K_a), and thermodynamic parameters of the reaction using Origin software. Calorimetric titration with all drugs was carried out at 25 °C. The titration for drugs **1**, **2**, and **5** was carried out at various temperatures in addition to 25 °C, and the change in heat capacity (ΔC_p) associated with the binding reaction was determined by the relationship $\Delta C_p = \Delta H/\mathrm{d}T$.

Supporting Information Available: Table of elemental analysis for **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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