

Entropy and Enthalpy in the Activity of Tubulin-Based Antimitotic Agents

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Abstract: Microtubules are important biological targets of antitumor chemotherapy. Tubulin polymerization inhibitors (TPIs) hinder polymerization whereas microtubule stabilizing agents (MSAs) promote tubulin polymerization and stabilize microtubules. The goal of enhancing binding affinity through favorable (positive) entropic contributions, a significant part of medicinal chemistry dogma, hinges on a rather simplistic assumption that ligand-protein binding interactions are primarily entropically driven. In turn, individual contributions of enthalpy and entropy to the overall potency of small molecules rarely are determined. Herein, we describe various antimitotic agents whose interactions with tubulin were explored and in which the individual enthalpic and entropic contributions were evaluated. These examples clearly demonstrate that the binding affinities of small molecules with their target proteins are more complex than often articulated; one should exercise caution when rationalizing the relative activity of these molecules and their analogues.

Keywords: Microtubules, antimitotic agents, tubulin, binding affinity, enthalpy, entropy.

MICROTUBULES AS TARGETS FOR ANTITUMOR THERAPY

Overview of Antimitotic Agents

Due to their integral role in cell division, microtubules and their constituent protein, tubulin, remain popular targets for cancer chemotherapy. A number of tubulin binding drugs commonly are used in cancer chemotherapy and many are in different phases of clinical trials. All of these drugs are classified as antimitotic agents, owing to their ability to inhibit the formation of the mitotic spindle.

There are two broad classes of these antimitotic agents: those that disrupt microtubule formation (tubulin polymerization inhibitors, TPIs); and those that stabilize microtubules (microtubule stabilization agents, MSAs). Albeit the effects of these two classes of antimitotic agents are drastically different, their modes of action are similar in that they both involve binding of the drug to a site on the tubulin dimer. The former class of antimitotic agents bind to unassembled tubulin dimers and subsequently inactivate them; the latter class of antimitotic agents bind to microtubules and stabilize them, ultimately blocking their dynamics [1].

Tubulin, Microtubules, and Dynamic Instability

Microtubules are ubiquitous, nonmembraneous cellular organelles that are essential for maintaining apposite cellular functions and structures. In interphase cells, microtubules are imperative to: intracellular vesicular transport (e.g., movement of secretory vesicles, endosomes, lysosomes); movement of cilia and flagella; cellular elongation and movement (migration); and maintenance of cell shape, particularly its asymmetry. In cells undergoing mitosis and meiosis, microtubules are necessary for the attachment of chromosomes to the mitotic spindle and their subsequent movement.

Structurally, microtubules are nonbranching, rigid, hollow tubes of protein that possess the ability to disassemble in one location and to reassemble in another location. The monomeric subunit of the microtubule polymer is tubulin, which is a 110kDa, heterodimeric protein, that is formed when α -tubulin and β -tubulin dimerize (Fig. 1). α - and β -tubulin isoforms typically share >88% amino acid sequence similarity; this homology is conserved among plants, animals, protists, and fungi [2, 3]. In addition to the α - and β -tubulin isoforms, various other tubulin isoforms have been recognized and have been implicated in chemotherapeutic drug resistance [4-7]. The most successful and potential anticancer agents bind to a site within β -tubulin molecule or at the $\alpha\beta$ -tubulin interface.

Typically 13 $\alpha\beta$ -tubulin dimers comprise a single protofilament, although shorter and longer protofilaments have been observed. Lateral interactions enable individual protofilaments to associate side by side into a microtubule cylinder. Microtubules measure 20 to 25nm in diameter and range in length from picometers to nanometers.

There are two populations of microtubules: stable, long-lived microtubules and unstable, short-lived microtubules. The former are found in interphase cells whereas the latter are found in cells wherein there is a need for rapid assembly and disassembly of cellular structures, such as during mitosis. In a cell undergoing mitosis, the characteristic interphase microtubule network disappears, and its constituent tubulin is used to form the spindle-shaped apparatus, which equally partitions chromosomes into daughter cells. Reformation of the interphase microtubule network occurs upon mitotic cessation.

Above a critical concentration (C_c) of tubulin subunits polymerize into microtubules; below the C_c , microtubules depolymerize. The transition to depolymerization is termed "catastrophe" whereas the transition to polymerization is termed "rescue". The end of the microtubule that undergoes assembly preferentially is termed the (+)-end whereas the end that assembles more slowly is termed the (-)-end. Nota-

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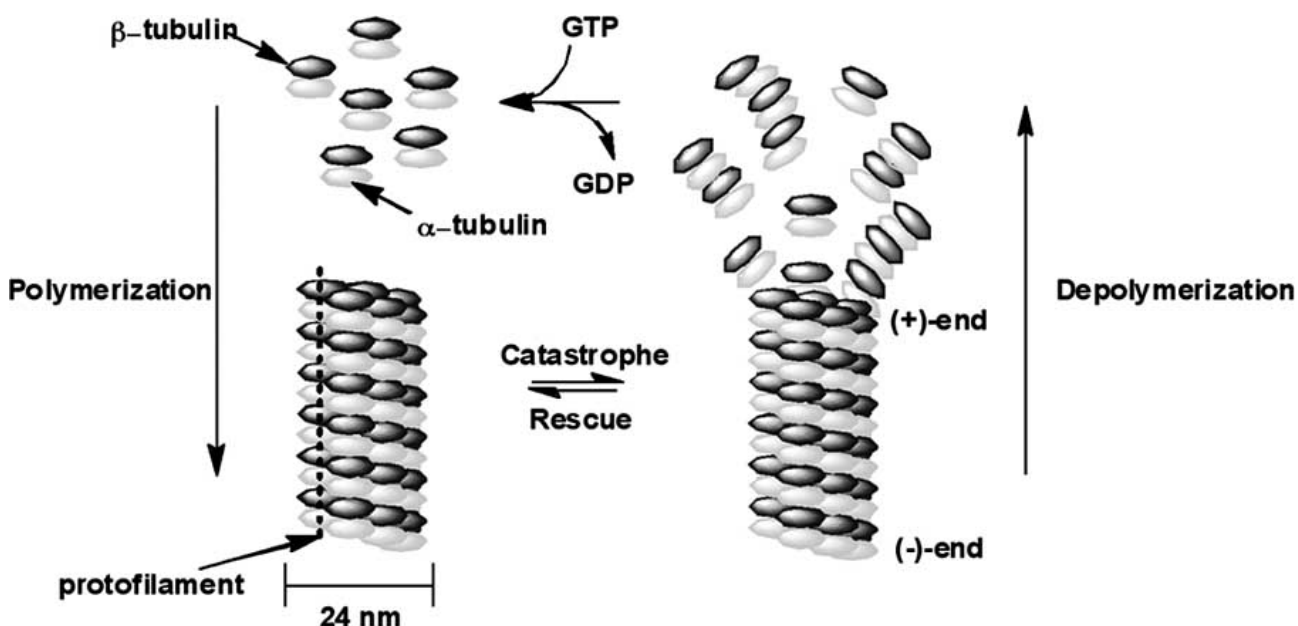


Fig. (1). Graphic depiction of microtubule formation and dynamics modified from [8] and [9].

bly, individual microtubules are in constant flux. That is, two properties intrinsic to all microtubules are dynamic instability, wherein microtubules oscillate between growth and shortening phases; and treadmilling, wherein subunits add to one end and dissociate from the other end. The effects of antimitotic agents on microtubule dynamics recently have been reviewed [7, 10, 11].

Alteration of microtubule dynamics as well as perturbation of mitosis at concentrations wherein they do not affect the polymer mass are two characteristics intrinsic to all known, effective microtubule binding drugs. However, all microtubule toxins alter microtubule mass as well as accumulate inside cells. It appears that both properties of microtubule binding drugs are essential to mitotic block and, possibly, there are stages in which one of these properties dominates over the other. It appears that both properties of microtubule binding drugs are essential to mitotic block and, possibly, there are stages in which one of these properties dominates over the other. Recently, Sengupta and Thomas [12] summarized the binding and cytotoxicity data of tubulin interacting drugs. Their review highlights the mechanistic differences between different tubulin interacting drugs. Our complementary analysis of the thermodynamic binding parameters for these compounds questions classic dogma regarding medicinal chemistry-lead optimization.

Tubulin Polymerization Inhibitors

Microtubule inhibitors are an established and an important group of cancer chemotherapeutic agents with clinical applications in the treatment of a multitude of cancer types, either as single agents or as part of different combination regimens [13]. There are two major binding sites for TPIs: the colchicine binding site and the vinca alkaloid binding site. The alkaloid colchicine is the oldest tubulin-binding drug to be studied in detail but several other TPIs have been shown to interact at this site, including podophyllotoxin, nodazole, sulfonamides, and several others [14-26].

The vinca alkaloids, vinblastine and vincristine, have been utilized successfully for some time in the treatment of various cancer types. Semi-synthetic analogues have been developed, several of which are used in the treatment of cancer or are in late stage clinical trials [27-29]. Various other TPIs, whose structures deviate from those of the vinca alkaloids, are suspected to bind within the vinca alkaloid binding site [30-39].

Microtubule Stabilizing Agents

For a major part of the last century, TPIs dominated the field of antimitotic; the introduction of MSAs into clinical practice constitutes a relatively recent development in cancer chemotherapy. The first MSA to receive FDA approval was the natural product paclitaxel (or Taxol®, Bristol-Myers-Squibb) in 1992; four years later, a closely related semi-synthetic analog, docetaxel (Taxotere®, Sanofi-Aventis U.S. LLC), received approval as a MSA chemotherapy [40].

In 1979, Susan Horwitz and co-workers demonstrated that paclitaxel promotes the *in vitro* assembly of microtubules in the absence of GTP, which normally is required for polymerization; and the microtubules that are formed are stable under destabilizing conditions (i.e., cold temperature, Ca^{2+} , and dilutions) [41]. *In vivo*, paclitaxel induces stabilization prototypical of microtubule bundles [42]. Stemming largely from the commercial success of Taxol®, a plethora of structurally diverse natural products, derived from various sources (e.g., corals, marine sponges, bacteria, and plants), have been shown to exhibit microtubule-stabilizing properties [1, 8].

MSAs are subdivided into three categories according to their point of contact with tubulin. The first group of compounds encompasses those compounds that bind within the paclitaxel site in microtubules. The same paclitaxel site, or an overlapping site, is described for the binding of paclitaxel and docetaxel as well as for epothilones, eleutherobin, discodermolide, sarcodictyins A and B and dictyostatin,

among others [43-48]. The structural diversity of the aforementioned molecules indicates the promiscuous nature of the paclitaxel binding site; nonetheless, numerous common pharmacophore models have been proposed, primarily based on conjecture [49-54].

Conversely, a separate, distinct binding site has been proposed for marine-derived polyketides, laulimalide and peloruside A [55, 56]. Moreover, a third group of compounds that have microtubule stabilizing capabilities but, whose binding to microtubules, as of yet, has not been detailed. This cluster of compounds includes, but is not limited to: taccalonolides A and E [57]; dicumarol and its analog coumadin, which also active coagulant agents [58, 59]; jatrophane polyester [60]; and lonafib (Sch66336), which is a transferase inhibitor that also was shown to bundle microtubules in cells and may act via inhibition of tubulin deacetylase [61].

THERMODYNAMICS OF SMALL MOLECULES BINDING TO PROTEINS

Gibbs Free Energy of Binding

The binding of small molecules to proteins is described in terms of the Gibbs free energy of binding ($K_a = e^{-(\Delta G/RT)}$). In turn, binding is dependent upon changes in the enthalpy (ΔH) and the entropy (ΔS) associated with binding ($\Delta G = \Delta H - T\Delta S$). The values of these imperative thermodynamic parameters rely upon the state of the system; values measured *in vitro* under one set of conditions only are valid under those conditions, which must be explicitly specified [62]. Extrapolation of the *in vitro* values to *in vivo* conditions is difficult, given that *in vivo* solution components, water activity, and concentration of other molecules are dissimilar and, often, unknown.

Enthalpic and Entropic Contributions to Binding

Enthalpic contributions to the overall binding affinity primarily arise due to variations in bonding, which, for non-covalent complexes, comprise short range interactions, including van der Waals forces, hydrogen bonding, and charge interactions [62]. The formation of new interactions ensues with a favorable (negative) enthalpic change whereas disruption proceeds with an unfavorable (positive) enthalpic change.

Entropic contributions to the overall binding affinity reflect the changes in degrees of freedom in the system, which encompass the rearrangements of solvent molecules or counter ions and rotational and translational changes in binding partners [62]. An increase in the number of degrees of freedom of the system causes a favorable (positive) entropic change. Accordingly, the binding of a small molecule to a protein should result in an overall negative entropic change due to a loss in degrees of freedom; however, in many instances, ΔS is large and positive due to the role of water and ions. Clearly, an infinite number of enthalpy and entropy values can afford the same Gibbs free energy of binding [63] and, thus, the binding affinity of a compound may be augmented by generating a favorable binding enthalpy, favorable solvation entropy, or by minimizing the unfavorable conformational entropy.

Enthalpy-Entropy Compensation

Extremely high affinity of a ligand for its target protein requires a combination of favorable entropic and enthalpic interactions [64]. Concurrent optimization of enthalpic and entropic interactions is complicated by the so-called *enthalpy-entropy compensation phenomenon*; that is, introduction of different chemical functionalities during affinity optimization frequently is accompanied by various enthalpic or entropic penalties [65]. Enthalpic optimization of the binding affinity is more difficult to achieve and often is accompanied by significant entropic penalties. Moreover, the addition of polar groups in an effort to enhance enthalpic contributions will be accompanied by a penalty resulting from necessary desolvation [66].

Enthalpy-entropy compensation is associated with solvent reorganization, which often accompanies protein-ligand interactions [67-71]. A plot of ΔH versus $T\Delta S$ that exhibits a linear relationship, with a slope equal to one, is indicative of complete compensation.

Since enthalpic optimization is complicated by both enthalpic and entropic penalties, lead optimization typically focuses on optimization of the entropic contribution to binding. Hydrophobic interactions are proportional to the number of nonpolar groups buried within the solvent and are the primary favorable contributors to the overall binding affinity; the common trend is to increase the hydrophobicity of drug candidates [72, 73]. Entropy optimization also encompasses conformationally constraining and pre-shaping molecules to the geometry of the binding site. The subsequent discussion will demonstrate that evaluation of both the enthalpic and the entropic contributions to the overall binding affinity is imperative; there is a limit to entropy optimization of a given drug candidate and, thus, it becomes crucial to introduce favorable enthalpic interactions in order to achieve nanomolar and subnanomolar affinities [63].

THERMODYNAMICS OF MICROTUBULE INHIBITING AGENTS BINDING TO β -TUBULIN

Compounds Binding within the Colchicine Binding Site

Colchicine compound (**1**) is a plant alkaloid that originally was isolated from *Colchicum autumnale* and *Gloriosa superba* [13]. Colchicine has been used in the treatment of gout and familial Mediterranean fever [75, 76]; while colchicine is cytotoxic against several cancer cell lines, toxicity issues impede its applicability as a cancer chemotherapeutic agent. However, several colchicine-site binders have attracted attention as potential anticancer agents. Colchicine and some of its analogs compounds (**2**) and (**3**) are efficient antimitotic agents (**15**) and are cytotoxic towards several cancer cell lines, as evidenced by their half minimal inhibitory concentration (IC_{50}) values (Table 1).

Colchicine inhibits microtubule assembly by binding to a high affinity site on β -tubulin (Fig. 2) [77, 78]. Colchicine binding occurs in a nearly irreversible manner and exerts a conformational change in tubulin as well as in colchicine itself [79, 80]. Notably, the level of tubulin isotypes that are present after preparation affects the colchicine binding. In contrast to colchicine, (Fig. 3), the binding of some colchicine analogs (e.g., DAAC, AC) occurs in a reversible manner [81].

Table 1. Cytotoxicity Exhibited by Some Compounds that Bind Within the Colchicine Binding Site

	IC ₅₀ A549 (nM)	IC ₅₀ HeLa (nM)	IC ₅₀ MCF7 (nM)	Mean IC ₅₀ (nM)	Ref.
Colchicine	16.86 ± 0.63	24 ± 11	108	-	[12, 82]
Podophyllotoxin	12	-	1	-	[12, 83, 84]
Nocodazole	-	3200	-	-	[12]
E7010	-	-	-	178	[12, 85]

The thermodynamic binding parameters of colchicine as well as of some colchicine analogs were elucidated from fluorescence anisotropy experiments (Table 2). While titration calorimetry is a more direct and straightforward method for elucidating thermodynamic binding parameters, the slow time-dependent binding exhibited by colchicine precludes this method [86]. The thermodynamic data on the colchicine analogs imply that the entropic contribution to the binding parameters is not significantly affected by the presence of the B-ring; however, an amino-substituent at the C-7 position of the B-ring was shown to convert an enthalpy-driven reaction into one that is entropy-driven [87].

Podophyllotoxin (**5**), isolated from the roots of the plant *Podophyllum peltatum*, has been shown to display cytotoxicity towards cancer cells (Table 2) [90]; however, while podophyllotoxin commonly is used in the treatment of genital warts, it is clinically toxic for cancer chemotherapy [13]. Podophyllotoxin effectively inhibits microtubule assembly by binding to the colchicine binding-site on β -tubulin [16]; the X-ray structure of podophyllotoxin bound within tubulin has been disclosed [77]. Podophyllotoxin binding differs from that of colchicine in that podophyllotoxin binding occurs rapidly, reversibly, and is not dependent on temperature. Moreover, podophyllotoxin does not appear to affect protein conformation.

Given the rapid binding of podophyllotoxin within β -tubulin, it was possible to use isothermal titration calorimetry (ITC) to determine its thermodynamic binding parameters (Table 2). The data reveal that podophyllotoxin binds in a manner that is comparable to colchicine and some of its analogs [86].

Sulfonamides are well-established, effective antibiotics, hypoglycemics, diuretics, and hypertensives. Their diverse

biological activity incited research efforts to discover a novel class of antitumor sulfonamides. E7010 compound (**11**), among others, demonstrated activity as a cancer chemotherapeutic and prompted clinical trials as orally active compounds [20, 24]. E7010 was shown to inhibit tubulin assembly upon binding to the colchicine binding site [85].

The success of E7010 led to the development of a series of second generation drugs with the indole scaffold in place of the pyridine ring. These second-generation drugs also reversibly bind to tubulin at the colchicine binding site [89] and exhibited TPI activity, with IC₅₀ values in the low micromolar range, with an approximate rank order of B \approx E > A \approx C \approx D [91-93].

The thermodynamic parameters of these second generation sulfonamide TPIs were determined using ITC (Table 2). The binding parameters of sulfonamides A – E revealed some interesting insights into their binding interactions within tubulin. Sulfonamide A did not exhibit enthalpy-entropy compensation, since all the thermodynamic parameters were highly temperature dependent. Notice, while the binding of sulfonamide C is entropically driven, it is approximately as cytotoxic as sulfonamide D, whose binding is enthalpically driven.

ΔS values for sulfonamides B and E were shown to vary in a compensatory manner with their respective ΔH values, more extensively for sulfonamide E than for sulfonamide B. The plot of ΔH against $T\Delta S$ for sulfonamide E displayed a linear relationship (slope = 1.04); the plot of ΔH against $T\Delta S$ for sulfonamide B also displayed a linear relationship (slope = 0.625). Therefore, sulfonamide B undergoes complete enthalpy-entropy compensation and sulfonamide D undergoes partial enthalpy-entropy compensation.

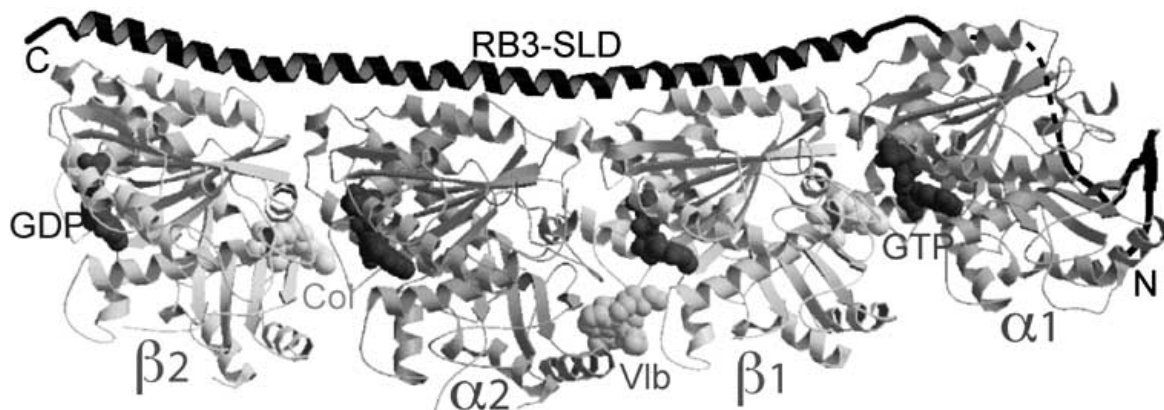
**Fig. (2).** The location of vinblastine in (Tc)₂R. (Tc)₂R-bound vinblastine (Vlb) is shown as a space filling model; the complex consists of RB3-SLD and two tubulin $\alpha\beta$ -heterodimers, with colchicine (Col) bound to the β -subunits at the interface with the α -subunit [74].

Table 2. Thermodynamic Parameters of Compounds Binding Within the Colchicine Binding Site of Tubulin. Parameters were Measured at 37°C Unless Otherwise Noted

	$K_a \times 10^{-3}$ (M^{-1})	ΔG ($kJ mol^{-1}$)	ΔH ($kJ mol^{-1}$)	ΔS ($J mol^{-1} K^{-1}$)	Ref.
Colchicine (1) [§]	215 ± 0.17	-42 ± 0.08	-26 ± 13	68 ± 7.1	[88]
NH ₂ -DAAC (4) [§]	3.57 ± 2.5	-31.38 ± 1.26	27.86 ± 4.18	194.2 ± 14.2	[87]
DAAC (3) [§]	27.93 ± 4.18	-35.98 ± 2.09	-15.40 ± 2.09	66.11 ± 6.69	[87]
AC (4) [§]	6.21	-33.1	-28.5	15.1	[81]
Podophyllotoxin (5)	9.78	-33.55	-39.15	-18.05	[86]
Sulfonamide A (6) [§]	0.30 ± 0.24 (at 25°C)	-25.35 ± 1.05	-7.53 ± 2.01	61.50 ± 6.78	[89]
Sulfonamide B (7) [§]	2.72 ± 0.37 (at 25°C)	-31.00 ± 0.17	-20.29 ± 0.33	36.40 ± 1.00	[89]
Sulfonamide C (8) [§]	0.16 ± 0.14 (at 25°C)	-23.72 ± 1.17	-3.64 ± 1.30	69.45 ± 4.31	[89]
Sulfonamide D (9) [§]	0.75 ± 0.20 (at 25°C)	-27.78 ± 0.33	-65.40 ± 4.10	-125.60 ± 13.5	[89]
Sulfonamide E (10) [§]	4.09 ± 1.63 (at 25°C)	-31.97 ± 0.50	-8.24 ± 0.33	80.33 ± 1.05	[89]

[§]For consistency, units have been converted to joules.

Compounds Binding within the Vinca Alkaloid Binding Site

The vinca alkaloids, vinblastine and vincristine compounds (12) and (13), respectively, are derived from the periwinkle plant, *Catharanthus roseus*, and are noted as the most successful anticancer agents within the past few years [94]. The success of these natural products, (Fig. 4), led to

the development of several semi-synthetic vinca alkaloid derivatives, (Fig. 4). Vinorelbine (14) is one semi-synthetic analog that already is in clinical use [28, 29]. The vinca alkaloids are purported to stabilize microtubule spindle dynamics. *In vitro*, substoichiometric levels of vinblastine were shown stabilize microtubules, possibly owing to binding at microtubule ends and inhibiting hydrolysis of GTP, such that

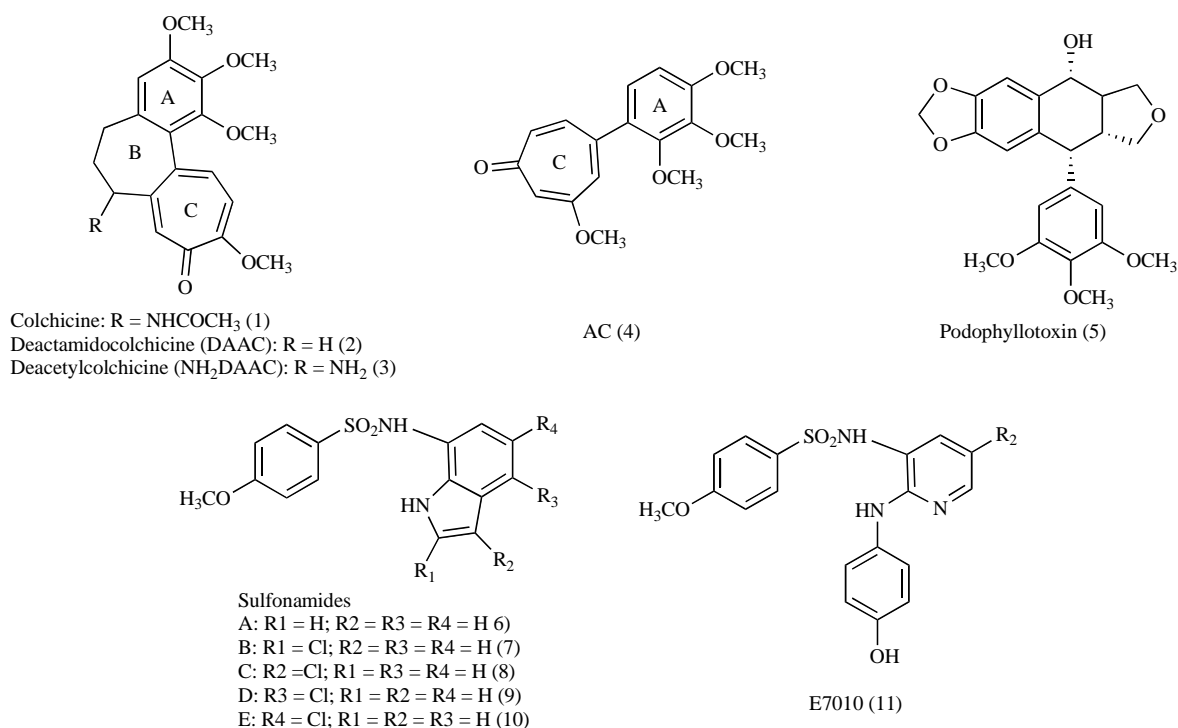
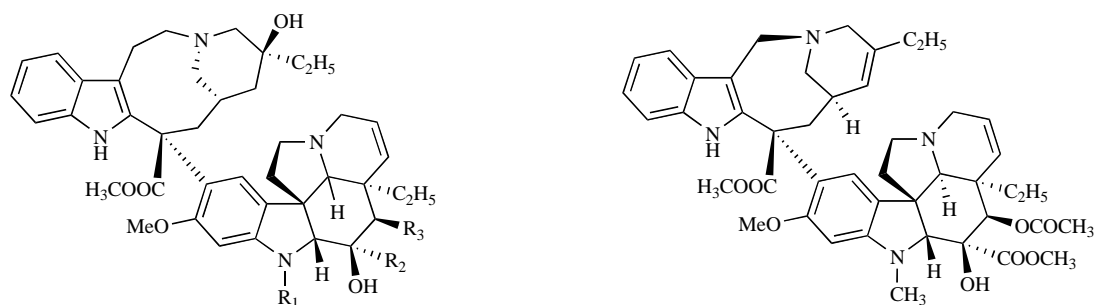


Fig. (3). Some compounds binding within the colchicine binding site of tubulin.



Vincristine: R1 = CHO; R2 = COOCH₃; R3 = OCOCH₃ (12)
 Vinblastine: R1 = CH₃; R2 = COOCH₃; R3 = OCOCH₃ (13)

Vinorelbine (14)

Fig. (4). Compounds binding within the vinca alkaloid binding site for which the thermodynamic binding parameters have been determined.

the microtubules do not lengthen or shorten at an appreciable rate and, as previously noted, microtubule dynamics are essential to mitotic progression [95-97].

Binding of the vinca alkaloids to β -tubulin occurs rapidly and reversibly at an intermolecular contact point (Fig. 2) [74, 98, 99]. In addition to the one high affinity site per tubulin dimer, there are several low affinity sites [100]. Similar to other antimetabolic agents, binding of the vinca alkaloids to β -tubulin induces a conformational change in tubulin itself; this conformational change promotes tubulin self-association in the presence of these TPis. Resultantly, at substoichiometric concentrations, spiral polymers and paracrystals are formed [101, 102]. The presence of tubulin isotypes does not appear to affect the binding of vinblastine to the protein [103]. Moreover, vinblastine was shown to bind to microtubules directly at their extremities and with high affinity [104, 105].

The thermodynamic binding parameters of vincristine, vinblastine, and vinorelbine were derived from sedimentation velocity data (Table 3). Vincristine exhibits the highest overall binding affinity (K_1K_2) whereas vinorelbine exhibits the lowest (vincristine > vinblastine > vinorelbine). The affinities of all three vinca alkaloids for tubulin, K_1 , were analogous; the differences in binding were attributed to the affinity of liganded heterodimers for polymers, K_2 . Vincristine, vinblastine, and vinorelbine displayed an increased maximum $\bar{s}_{20,w}$ with increasing temperature [5, 25, and 36°C], which implies an entropically driven reaction for the overall process. An entropically driven reaction is further supported by the ΔH^0 and ΔS^0 values; ΔS^0 values compensate for unfavorable ΔH^0 values. Markedly, the ΔG^0 values for these three vinca alkaloids only weakly correlate with their cytotoxicity profiles, (Table 4).

THERMODYNAMICS OF MICROTUBULE STABILIZING AGENTS BINDING TO β -TUBULIN

Compounds Binding to the Paclitaxel Binding Site

Both paclitaxel, extracted from the bark of the pacific yew tree, *Taxus brevifolia*, and its semi-synthetic analog, docetaxel compounds (15) and (16), respectively [112], have achieved great clinical success for the treatment of a variety of cancers, including: ovarian, metastatic breast, head, neck, and lung cancers [113]. The taxoid class of MSAs bind to a single site on polymeric tubulin (Fig. 5) with high affinity and, in turn, stabilize microtubules; notably, binding to dimeric tubulin occurs with much weaker affinity [114]. The paclitaxel binding site is located in the so-called M-loop of β -tubulin.

There are several factors that limit to the general applicability of taxol and docetaxol, (Fig. 6): they have low aqueous solubility; they develop pleiotropic drug resistance mediated by overexpression of the P-glycoprotein; and mutations in β -tubulin have been shown to contribute to paclitaxel resistance [115-117]. The disadvantages associated with the taxanes incited a search for other natural products that mimicked their mechanisms of action, but exhibit more auspicious properties.

Epothilones A and B compounds (17) and (18), respectively are polyketide macrolides that were first isolated from the myxobacterium *Sorangium cellulosum* So ce90 [118, 119]. While markedly different in structure from the taxanes, the epothilones have a "paclitaxel-like" mechanism of action; that is, they bind to microtubules in the paclitaxel binding site and stabilize microtubules to virtually the same degree as paclitaxel [43]. Diaz reported the thermodynamic binding parameters of epothilones A and B binding within the paclitaxel binding (Table 5). Arguably, the epothilones

Table 3. Thermodynamic binding Parameters of Compounds Binding within the Vinca Alkaloid Binding Site

	$K_1K_2 \times 10^{-10}$ (M ⁻¹) at 25°C	ΔG^0 (kJ mol ⁻¹)	ΔH^0 (kJ mol ⁻¹)	ΔS^0 (J mol ⁻¹ K ⁻¹)	Ref.
Vincristine (12)	5.2	-61.09	-15.48	154.8	[106]
Vinblastine (13)	2.3	-58.99	36.4	322.17	[103]
Vinorelbine (14)	2.1	-58.99	30.5	301.25	[106]



Fig. (5). The Taxol binding site in β -tubulin, which is located at the so-called M-loop [1].

represent the most complex natural product to undergo extensive SAR analyses [120-126].

Comparison of the IC_{50} values of epothilones A and B with those of paclitaxel and docetaxel reveals that their cancer cell cytotoxicities rival each other (Table 6). Importantly, the epothilones are poor substrates for the P-glycoprotein efflux pump, retain potent activity against P-glycoprotein-overexpressing, paclitaxel-resistant cells, and are more water-soluble than the taxanes [43, 127, 128]. Epothilones A and B also are active against paclitaxel-resistant cells, which display reduced sensitivity to paclitaxel due to tubulin mutations [115].

Discodermolide (**21**) was extracted from the marine sponge *Discodermia dissoluta* and is a poly-propionate-derived polyhydroxy- δ -lactone [131, 132]. Original interest in discodermolide was based upon its immunosuppressive activity; however, it was soon recognized as a nanomolar inhibitor of human cancer cells *in vitro*. Discodermolide later was shown to stabilize microtubules and induce microtubule assembly to a greater extent than paclitaxel [46, 133, 134]; however, its cytotoxicity is less than that of the taxanes as well as of epothilones A and B (Table 6). Several discodermolide analogs have been synthesized; unfortunately, they were found to have both diminished antiproliferative activity and microtubule stabilization activity [135]. Discodermolide competitively inhibits paclitaxel binding to microtubules [46, 128, 130, 136] and the thermodynamic binding parameters have been determined (Table 5).

To date, discodermolide remains one of the most potent inducers of microtubule assembly; dictyostatin (**20**) is

Table 4. Cytotoxicity of Compounds Binding within the Vinca Alkaloid Binding Site for which the Thermodynamic Binding Parameters have been Determined

	IC_{50} A549 (nM)	IC_{50} HeLa (nM)	IC_{50} MCF7 (nM)	IC_{50} L1210 (nM)	Ref.
Vincristine	2.31 ± 0.08	0.95 ± 0.08	0.98	20	[21, 107-109]
Vinblastine	53.7 ± 2.9	-	-	0.5	[109, 110]
Vinorelbine	-	-	500 ± 130^e	170^y	[109, 111]

Table 5. Thermodynamic Binding Parameters of Compounds Binding within the Paclitaxel Binding Site of Tubulin. Parameters were Measured at 37°C Unless Otherwise Noted

	$K_a \times 10^{-7}$ (M^{-1})	ΔG^0 ($kJ mol^{-1}$)	ΔH^0 ($kJ mol^{-1}$)	ΔS^0 ($J mol^{-1} K^{-1}$)	Ref.
Paclitaxel (15)	1.07 ± 0.11	-41.7 ± 0.2	-51 ± 4.2	-29.3 ± 13	[129]
Docetaxel (16)	3.09 ± 0.22	-44.4 ± 0.2	-52.5 ± 2.3	-25.5 ± 7.5	[129]
Epothilone A (17)	2.93 ± 0.44	-44.3 ± 0.4	-65.6 ± 2.4	-68.3 ± 7.9	[129]
Epothilone B (18)	60.8 ± 10	-52.1 ± 0.4	-70.7 ± 7	-59.7 ± 22.8	[129]
tmt-epo B (19)	194 ± 33	-55.4 ± 0.6	-37 ± 11	62 ± 37	[129]
Dictyostatin (20)	16.8 ± 2.0	-48.8 ± 0.3	-45.9 ± 2.6	9.5 ± 8.5	[130]
Discodermolide (21)	526 ± 72	-57.7 ± 0.3	-33.2 ± 7.6	79.5 ± 2.5	[130]
Eleutherobin (22)	1.36 ± 0.62	-43.6 ± 0.03	-33.2 ± 3.2	32.9 ± 10.3	[130]
Sarcodictyin A (23)	0.16 ± 0.06	-36.8 ± 0.8	-23.6 ± 2.6	43.12 ± 8.6	[130]
Sarcodictyin B (24)	0.20 ± 0.07	-37.9 ± 0.7	-29.3 ± 3.1	27.1 ± 9.9	[130]
Estradiol analog (25)	0.047 ± 0.013	-33.4 ± 0.8	-18.2 ± 6.8	51.6 ± 22.1	[130]

somewhat more effective in the process. Discodermolide is significantly more active than paclitaxel against different types of different multidrug-resistant cells lines; however, its ability to overcome P-glycoprotein mediated multidrug resistance is less than that of the epothilones [46, 128, 137]. Discodermolide is active in cells, which are resistant to paclitaxel and epothilones due to specific tubulin mutations. Synergistic effects have been reported for the combination of discodermolide with paclitaxel in the growth inhibition of various cancer cell lines, both *in vitro* and *in vivo* [137, 138]. Unfortunately, the combination discodermolide and paclitaxel is most potent at concentrations that give rise to drug-induced aneuploidy rather than mitotic arrest [138].

Eleutherobin compound (**22**), a tricyclic diterpene, was isolated from the soft coral *Eleutherobia* sp. off the coast of Western Australia [139]. Long *et al.* demonstrated that eleutherobin is a potent inhibitor of tubulin polymerization *in vitro* in the absence of GTP [140]. At 37°C, the tubulin polymerization activity of eleutherobin is lower than paclitaxel; at 21°C, tubulin polymerization of eleutherobin is greater than that of paclitaxel. Treatment of cancer cells with eleutherobin afforded all the characteristics associated with microtubule inhibitors and the morphological changes at the cellular level essentially are indistinguishable from paclitaxel.

Eleutherobin competitively displaces paclitaxel from preformed microtubules with a K_i of 2.1 μM [140]. The thermodynamic binding parameters of eleutherobin binding within the paclitaxel binding site have been reported (Table 5). Like paclitaxel, eleutherobin induces homologous populations of long, rigid microtubules. Growth inhibition of human cancer cells with eleutherobin occurs in the nanomolar range (Table 6). Similar to paclitaxel, eleutherobin exhibits reduced activity against P-glycoprotein-overexpressing multidrug resistant cancer cells [45, 140, 141].

Sarcodytins A and B, (**23**) and (**24**), are close structural relatives of eleutherobin and may be regarded as unglycosy-

lated eleutherobin variants. Pietra and co-workers first isolated Sarcodictyins A and B from the Mediterranean stolonifer *Sarcodictyon roseum* [142]; others have reported the isolation of sarcodictyins A from the soft corals *Eleutherobia aurea* and *Bellaonella albiflora* [143, 144]. Other sarcodictyins variants have been isolated, but, as of yet, a limited amount of biological profiling has been reported [145].

Nearly 10-years after their isolation, sarcodictyins A and B were shown to exhibit "paclitaxel-like" properties. These sarcodictyins were found to bind within the paclitaxel binding site within β -tubulin and, later, their thermodynamic binding parameters were elucidated (Table 5). Sarcodictyins A and B do inhibit human cancer cell growth (Table 6). Perhaps more importantly, unlike eleutherobin, they retain most of their activity against P-glycoprotein-overexpressing, multidrug resistant cells and they were found to retain full activity against two paclitaxel-resistant sublines of 1A9 ovarian carcinoma cell line, which had undergone tubulin mutations [45, 146]. Various sarcodictyins analogs have been prepared by total synthesis, some of which exhibit improved biological profiles [45, 125, 147].

Dictyostatin, *cis*-CP-tmt-epo B, and the estradiol analog 3,17 β -diacetoxy-2-ethoxy-6-oxoB-homo-estra-1,3,5-triene compounds (**20**), (**19**), and (**26**), respectively, also are paclitaxel-binding MSAs. Their binding parameters have been determined (Table 5) and they appear to mimic the patterns observed for other paclitaxel site binding MSAs. Both dictyostatin and *cis*-CP-tmt-epo B exhibit cytotoxicities in the nanomolar range (Table 6).

Diaz measured the thermodynamic binding parameters *via* fluorescence anisotropy and correlated paclitaxel site binding with microtubule polymerization of twelve MSAs known to bind at this binding site in β -tubulin: paclitaxel, docetaxel, epothilone A, epothilone B, tmt-epo B, dictyostatin, discodermolide, eleutherobin, sarcodictyins A, sarcodictyins B, cyclostreptin, and an estradiol analog [129, 130]. Initial results indicated that Gibbs free energy of binding correlated well with IC_{50} values [129]. Additional studies revealed, ΔH of binding better correlated with IC_{50} values [130]. That is, compounds whose ΔS of binding were negative, or only slightly positive, and, thus, their binding was enthalpy driven, exhibited high cytotoxicity (e.g., paclitaxel, epothilones A and B); compounds with strongly positive entropic contributions (i.e., binding is more entropically driven) exhibited lower cytotoxicities (e.g., eleutherobin, discodermolide) than the former compounds. Markedly, both the former and the latter compounds exhibited high affinities for the paclitaxel binding site. Diaz's results gave rise to the suggestion that optimization of paclitaxel site drugs requires designing compounds with large, favorable enthalpic contributions to binding.

Conspicuously, of the MSAs included in Diaz's study, discodermolide may be viewed as the most conformationally flexible molecule and, thus, may be presumed to be the least entropically favorable molecule. Dictyostatin essentially is the cyclized form of discodermolide. One might assume that in its conformationally restricted form, dictyostatin binding would benefit from added entropic gain, thereby serving to increase binding affinity; however, the data indicates otherwise. This note further highlights the complexity associated with enhancing binding affinity via augmenting the enthalpic

Table 6. Cytotoxicity of Compounds Binding within the Paclitaxel Binding Site for which the Thermodynamic Binding Parameters have been Determined

	IC_{50} 1A9 (nM)	IC_{50} A549 (nM)	Ref.
Paclitaxel	1.0 \pm 0.4	1.4 \pm 0.2	[129]
Docetaxel	0.4 \pm 0.2	3.0 \pm 0.3	[129]
Epothilone A	4.1 \pm 1.0	7.5 \pm 1.4	[129]
Epothilone B	1.2 \pm 0.3	0.8 \pm 0.1	[129]
tmt-epo B	0.51 \pm 0.07	0.17 \pm 0.08	[129]
Dictyostatin	3.6 \pm 1.0	5.6 \pm 1.0	[130]
Discodermolide	17.6 \pm 2.7	6.6 \pm 0.8	[130]
Eleutherobin	13.2 \pm 7.0	3.3 \pm 0.2	[130]
Sarcodictyins A	32 \pm 21	36 \pm 12	[130]
Sarcodictyins B	103 \pm 18	50 \pm 15	[130]
Estradiol analog	43000 \pm 3000	>20000	[130]

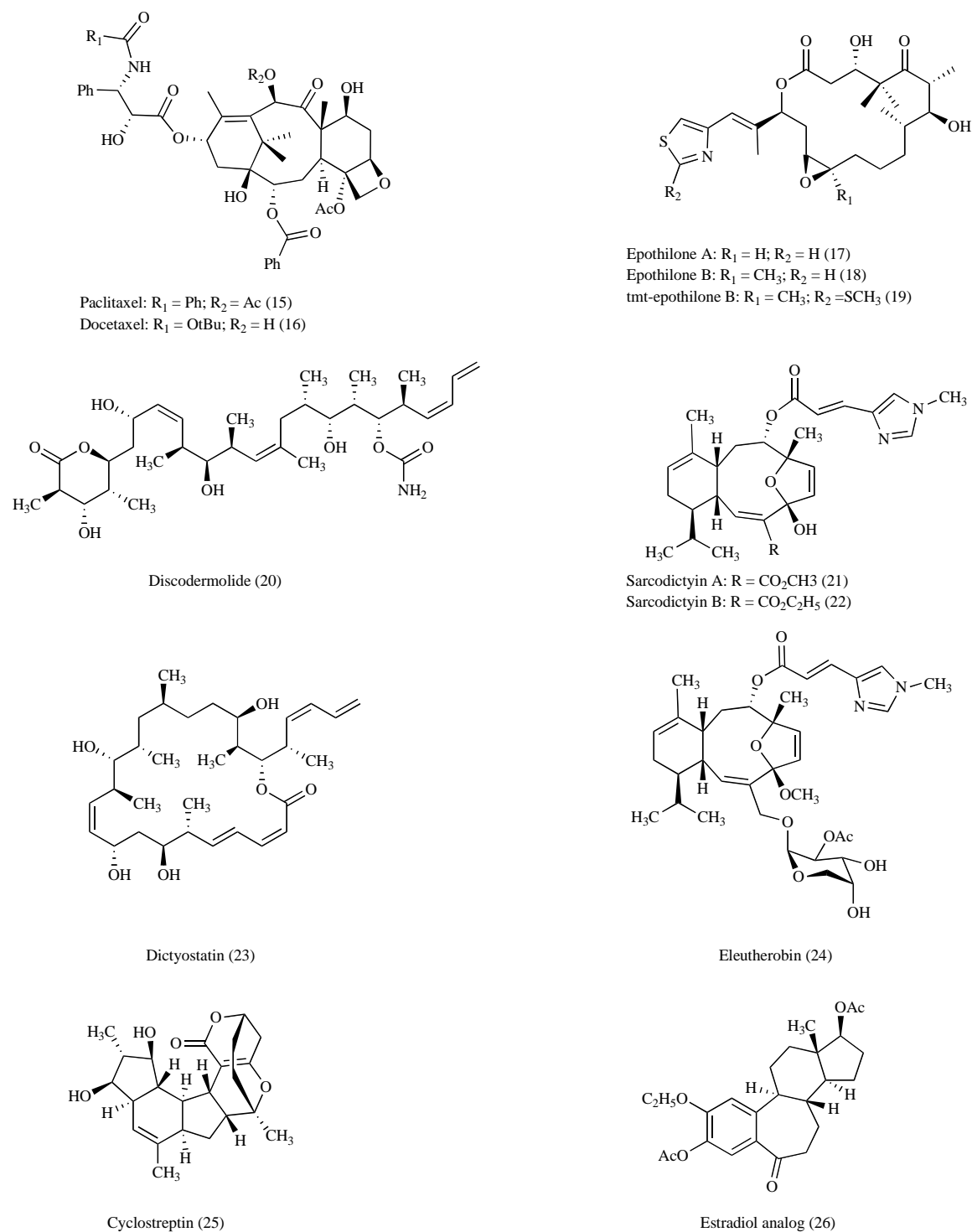


Fig. (6). Compounds binding within the paclitaxel binding site for which the thermodynamic binding parameters have been determined.

and the entropic contributions associated with a given molecule.

IMPLICATIONS OF ANTIMITOTIC AGENTS BINDING WITHIN TUBULIN

Conclusions that may be Drawn about Interactions of Antimitotic Agents with Tubulin

Antimitotic agents frequently used in cancer chemotherapy and their modes of action are described either as inhibit-

ing tubulin polymerization or as stabilizing microtubules. All of the antimitotic agents described above bind within the β -tubulin molecule or at the $\alpha\beta$ -tubulin interface at one of three sites: the colchicine binding site, the vinca alkaloid binding site, or the paclitaxel binding site. While they differ in their point of interaction with the protein and their modes of action, inhibition of human carcinoma cell proliferation ensues by blocking the formation of the mitotic spindle. Cancer cell cytotoxicities of these compounds often are in the nanomolar range.

Herein, the compounds presented were chosen because their thermodynamic binding parameters have been reported. These thermodynamic binding parameters adequately describe their binding affinities and, thus, afford a detailed description of the binding interactions of the aforementioned antimetabolic agents with tubulin.

Complexity of Binding Affinities of Antimitotic Agents

It is highly desirable to design new antimetabolic agents with improved specificity and affinity for tubulin. In order for these new antimetabolic agents to be useful pharmacologically, they must be selective for tubulin and not for other targets. As evidenced by the examples herein presented, such design of the Gibbs free energy of binding is more complex than has been historically articulated.

Balancing enthalpic and entropic contributions facilitates binding affinity maximization. Engineering enthalpic interactions is more difficult than engineering entropic interactions, as it is less energetically costly to introduce hydrophobic groups. Determining the thermodynamic binding parameters enables one to recognize the origin of the forces that contribute to a favorable Gibbs free energy of binding. With the thermodynamic binding parameters in hand it becomes possible to separate those compounds whose binding is entropically driven (i.e., binding arises as a result of exclusion from the solvent) from those compounds whose binding is enthalpically driven (i.e., binding arises as a result of establishing favorable interactions with tubulin).

Paclitaxel site binding appears to be enthalpically driven. Optimization of new and existing MSAs that target the paclitaxel site within β -tubulin may be difficult due to enthalpic and entropic penalties that are associated with enthalpic augmentation. Conversely, vinca alkaloid analog and derivative optimization may prove more facile, as, at 25°C, binding is entropically driven, since ΔH of binding is positive or only slightly negative.

Simplifying the principles of lead optimization by only focusing on the Gibbs free energy of binding or by solely enhancing the entropic contributions to binding will not suffice; dissection of the thermodynamic parameters of binding dictates the subsequent modifications required for optimization. However, the situation is further complicated by the fact that extrapolation of the thermodynamic data gathered *in vitro* to *in vivo* conditions often is difficult or impossible because *in vitro* and *in vivo* solution components, water activity, and concentration of other molecules are disparate.

SIGNIFICANCE

The biochemical interactions and the thermodynamic interactions between various antimetabolic agents with tubulin or with microtubules have been studied. The Gibbs free energies of these compounds have been dissected into individual enthalpic and entropic parameters. Historically, medicinal chemistry has simplified ligand optimization to focus efforts towards enhancing the overall binding affinity by amplifying the entropic contributions to binding, as entropic optimization is not encumbered by significant enthalpic penalties. However, the thermodynamic data herein presented demonstrates that ligand-protein interactions are complex and their optimization is facilitated by knowing whether binding is enthalpically driven or it is entropically driven.

Optimization of protein-ligand interactions via entropy optimization, when, in fact, binding is enthalpically driven, may prove fruitless, as Diaz suspects is the situation for paclitaxel binding site MSAs. All protein-ligand interactions are more complex than often is articulated; therefore, completely describing these interactions necessitates evaluation of all thermodynamic parameters, as has been done for the compounds herein presented.

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