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ARTICLE

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General features of the recognition by tubulin of colchicine and related compounds

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Abstract The kinetic mechanisms of the binding to tubulin of colchicine and eight different analogues have been studied to elucidate details of the recognition mechanism. All of the analogues follow a two step binding mechanism i.e. binding occurs via an initial step with low affinity, followed by an isomerisation of the initial complex leading to the final high affinity state. For several analogues the kinetic and thermodynamic data of both processes are compared here. For all the analogues the ΔG_1° of initial binding at 25 °C varies between –13.3 and –28.8 kJ · mol^{-1}. For the second step ΔG_2° varies between –2.4 and –27 kJ · mol^{-1}. These limited ranges of free energy change are, however, obtained by a great variety of enthalpy changes and compensatory entropy changes. Comparison of the data for the first and second steps indicates that structural alterations of the drugs always change the thermodynamic parameters of the two steps, and the changes in the first and the second steps are in opposite directions. The fact that this range of experimental behaviour can be incorporated into a general mechanism encourages the extension of these investigations to other colchicine analogues and related compounds with potential pharmaceutical applications.

Key words Colchicine · Tubulin · Cytostatics · Binding

Abbreviations *ALLO* Allocolchicine · *COL* Colchicine · *IDE* Colchicide · *ISO* Isocolchicine · *MTC* 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone · *TCB* 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl · *TKB* 2,3,4-trimethoxy-4′-acetyl-1,1′-biphenyl

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Introduction

Characterization of binding kinetics

Colchicine (COL) is well known as a cytostatic agent because of its effective inhibition of microtubule assembly and consequently of cell division. The cytostatic action is due to the fact that the tubulin-colchicine complex interacts with the ends of the microtubules (Margolis and Wilson 1977; Lambeir and Engelborghs 1980; Skoufias and Wilson 1992; Vandecandelaere et al. 1994, 1997). The mechanism of binding of colchicine to tubulin has been the subject of a large number of studies using many different approaches. Several recent reviews cover the behaviour of many colchicine derivatives with respect to their binding to tubulin, their cellular action and their spectroscopic properties (Brossi et al. 1988; Hamel 1990; Bane 1991). In this paper we will concentrate on quantitative studies that contributed to the understanding of the two step binding mechanism. In 1974 Bhattacharyya and Wolff demonstrated that the fluorescence of colchicine increases substantially upon its binding to tubulin. This observation allowed Garland to study the binding kinetics using a fluorescence stopped flow (Garland 1978). This kinetic study immediately revealed a number of peculiar aspects of the interaction between tubulin and colchicine:

(i) the binding kinetics are extremely slow. The apparent bimolecular rate constant is only 66 M^{-1}s^{-1} at 25 °C while diffusion limited values between 10^6 and 10^8 M^{-1}s^{-1} can be expected for small-molecule protein interactions (Fersht 1985);

(ii) the increase of fluorescence is described by a sum of two exponentials;

(iii) both observed rate constants increase in a nonlinear way with the concentration of colchicine, which can be interpreted in terms of a two step binding mechanism, comparable to the Michaelis-Menten mechanism of catalysis:

$$T + COL \overset{K_1}{\leftrightarrow} T \cdot COL \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} T \cdot COL^* \quad (1)$$

Where T · COL stands for the initial low affinity complex and T · COL* for the final high affinity state. K_1 is the binding constant for the initial complex, k_2 and k_{-2} are the rate constants for the subsequent isomerization in the forward and backward direction respectively. This mechanism gives rise to a nonlinear concentration dependence of the observed rate constant (k_{obs}):

$$k_{obs} = k_{-2} + k_2 \cdot (K_1 \cdot [COL])/(1 + K_1[COL]) \quad (2)$$

with k_2 usually being negligibly small. (Note that in the low concentration region, when $K_1 \cdot [COL] \ll 1$a linear relation is obtained, and k_{obs} behaves as a bimolecular rate constant).

The two exponentials observed with tubulin preparations from pig brains were shown to be due to the presence of tubulin isoforms that differ in their binding behaviour (Banerjee and Ludueña 1987). In bovine cerebral tubulin preparations, four isoforms have been identified and three have been isolated, purified and kinetically characterized: $\alpha\beta_{II}$, $\alpha\beta_{III}$ and $\alpha\beta_{IV}$. It has been shown that $\alpha\beta_{II}$ and $\alpha\beta_{IV}$ behave similarly with respect to their colchicine binding kinetics, while $\alpha\beta_{III}$ reacts more slowly (Banerjee and Ludueña 1987, 1991, 1992; Banerjee et al. 1994, 1997). Most kinetic stopped flow studies are performed on the natural mixture of isoforms but the rate constants of the two populations can nevertheless be obtained by fitting to the sum of two exponentials (individual isoforms are not resolved). We refer to these two populations as the fast- and slow-binding tubulin isoforms.

The study of Garland (1978) was expanded by Lambeir and Engelborghs (1981) using a stopped flow with an optical pathway of 2 mm, allowing the concentration range to the extended to 8 mM colchicine. In this way the observed deviation from linearity of k_{obs} vs. [COL] was much more pronounced and more accurate values of K_1 and k_2 could be obtained, at least for the fast-binding tubulin isoforms. The binding kinetics were also studied at different temperatures and from the temperature dependence the standard enthalpy change of the first step ($\Delta H^\circ_1 = -33 \pm 8$ kJ · mol^{-1}) and the activation energy of the second step ($\Delta E^{\#}_2 = 100 \pm 5$ kJ · mol^{-1}) could be determined. The initial binding step is clearly strongly exothermic.

The dissociation process of colchicine was studied by displacing it by podophyllotoxin and correcting for the parallel pathway of the loss of binding sites by the partial denaturation of tubulin (Díaz and Andreu 1991). Since the backward isomerization is rate limiting for dissociation, it is k_{-2} Eq. (1)) that is determined in this way. From the temperature dependence of k_{-2} the activation energy of the reverse isomerization ($\Delta E^{\#}_{-2}$) was found to be 114 ± 10 kJ · mol^{-1} and in this way the full thermodynamic pathway for the binding of colchicine to the fast-binding tubulin isoforms could be constructed. This technique had previously been successfully applied to other colchicine analogous (Engelborghs and Fitzgerald 1986, 1987). For colchicine and 9 analogues the binding constant K_1 (and therefore also ΔG°_1) have therefore been determined, and from the temperature dependence ΔH°_1 and ΔS°_1 (from the slope and the intercept of a plot of $\ln K_1$ vs. 1/T). For the second step not only K_2, ΔG°_2, ΔH°_2, ΔS°_2 are known but also $\Delta E^{\#}_2$ and $\Delta E^{\#}_{-2}$, the activation energies of the forward and the backward isomerisation, could be obtained from the Arrhenius plot ($\ln k_{obs}$ vs. 1/T) (See Table 1).

General implications of the two step mechanism. One can imagine the two-step binding mechanism of colchicine to be a particular example of the more general phenomenon of induced fit (Koshland et al. 1966): a fast initial binding of low affinity followed by a rearrangement leading to the final complex of high affinity and high specificity. In this way it is easy to imagine how ligand binding can lead to the emergence of new properties. Binding of a small molecule to a perfectly preformed binding site does not alter the conformation and therefore does not create any new property or any event. However, the achievement of full complementarity after an initial binding to a low affinity site can lead to a changed conformation of the protein with new properties (enzymatic activity, affinities for other proteins etc). These considerations point to the general implications of a thorough study of this binding mechanism.

Evidence for a conformational change in tubulin induced by colchicine binding. Many observations indicate the formation of a different conformation of tubulin after colchicine

Table 1 Thermodynamic data for the binding of different colchicine analogues to tubulin (f and s point to the families of fast and slow binding isoforms). ΔG° is calculated at 25 °C

	ΔG°_1	ΔH°_1	ΔS°_1	ΔG°_2	ΔH°_2	ΔS°_2
COLf[1,2]	–13.3 ± 0.3	–33 ± 12	–63 ± 40	–27 ± 0.5	6 ± 11	111 ± 36
MTCf[3]	–13.9 ± 0.3	–1 ± 1.6	43 ± 7	–17 ± 0.5	–23 ± 6	–20 ± 18
MTCs[3]	–17.5 ± 0.4	24 ± 7	142 ± 24	–13 ± 0.8	–35 ± 7	–74 ± 26
TCB[4]	–28.8 ± 0.3	44 ± 9	240 ± 31	–2.4 ± 0.5	–22 ± 11	–66 ± 38
TKB[4]	–22.3 ± 0.3	–40 ± 14	–60 ± 47	–8.6 ± 0.3	22 ± 5	103 ± 17
ALLOf[5]	–23.6 ± 0.3	68 ± 5	303 ± 17	–12 ± 0.2	–49 ± 21	–124 ± 70
ALLOs[5]	–25.0 ± 0.6	45 ± 30	224 ± 116	–8.7 ± 0.2	–26 ± 18	–58 ± 40
MDC[6]	–15.6 ± 0.2	49 ± 11	216 ± 33	–12 ± 0.2	–58 ± 14	–155 ± 47
IDE[7]	–21.2 ± 0.2	54 ± 7	251 ± 23	–9.6 ± 0.1	–78 ± 18	–229 ± 67

Units: ΔG° and ΔH° in kJ · mol^{-1}; Δs° in J · K^{-1} · mol^{-1}

Errors: The errors have been obtained from the curve fitting programme Sigmaplot^R. Our recent experience using Kinfit and global analysis of the kinetic curves, shows that the same rate constants are obtained, but with a tenfold reduced error (Díaz et al. 1997). It is therefore clear that the errors reported here are greatly overestimated.

References: 1. Lambeir and Engelborghs 1981; 2. Díaz and Andreu 1991; 3. Engelborghs and Fitzgerald 1986, 1987; 4. Dumortier et al. 1996a, 5. Dumortier et al. 1996b; 6. Dumortier et al. 1997b; 7. Dumortier et al. 1997a

binding: tubulin-colchicine shows enhanced GTP-ase activity (David-Pfeuty et al. 1979), the association of the tubulin monomers into the heterodimer is increased (Detrich et al. 1982), there are changes in the CD and Raman spectrum of the protein and the drug (Detrich et al. 1981, Andreu and Timasheff 1982 b, Audenaert et al. 1989, Chabin et al. 1990). Furthermore tubulin-colchicine shows an altered proteolysis pattern as compared to tubulin itself and altered reactivity towards alkylating agents (Ludueña and Roach 1981).

The binding of colchicine to tubulin is therefore a good model system because the equilibrium constants for the two steps can be determined. General questions then arise e.g. what are the forces involved in the two steps and are different parts of the molecule involved in different steps? What are the hinges and levers of the conformational change? How far does the conformational change extend and is there a linkage between the extent of the conformational change and the equilibrium constant of the second step? While the answer to some of these questions is dependent on the knowledge of the detailed structure of both tubulin and colchicine, the answer to some other questions can come from experiments with analogues of colchicine.

The relation between the two steps and two subsites of binding. The low affinity of the first step suggests the possibility that only a part of the molecule interacts with the protein in this initial step. In the second step, coupled to the conformational change of ligand and protein, the rest of the drug molecule could then interact with the protein. As with the drug, the binding site on the protein can also be divided into two subsites, one for the first part and one for the second part. This model was first proposed by Andreu and Timasheff (1982 a, b) and naturally leads to the following question: which part of the drug molecule interacts in the first step with the first subsite of tubulin, and which part interacts in the second step with the second subsite. To answer these questions, three types of experiments have been performed: (i) equilibrium binding studies of single ring analogues and comparison of the thermodynamic binding parameters with those of the parent molecule, (ii) kinetic competition studies of these single ring analogous with the parent molecules, and (iii) equilibrium and kinetic studies of whole-colchicine analogues with subtantial or minor modifications in one of the different rings.

Over 200 colchicine analogues have been prepared and their tubulin interactions, cytotoxicity and antitumor activity have been tested (Capraro and Brossi 1984). However, in this review only those compounds will be considered that were quantitatively studied and that shed some light on the question of the contribution of different parts of the drug molecule to the characteristic properties of the different steps of the binding mechanism.

Equilibrium binding studies of single ring analogues of colchicine

The first experimental approach to answer this question was made by Andreu and Timasheff (1982 a), who studied the equilibrium binding of single ring analogues of colchicine: i.e. NAM (N-acetyl-mescaline) as an analogue of the A-ring and TME (tropolone-methyl-ether) as a C-ring analogue, using the Hummell-Dryer technique of column chromatography and radioactive molecules for detection. The binding of the single rings was studied as a function of temperature and the $\Delta H°$ of TME was found to be -34.7 ± 4 kJ $\cdot$ mol^{-1} which corresponds rather well with the $\Delta H°$ for the initial binding of colchicine (-33 ± 12 kJ $\cdot$ mol^{-1}, Lambeir and Engelborghs 1981). The binding of NAM was predicted to be endothermic (Andreu and Timasheff 1982 a). These data therefore strongly suggest that the C-ring of colchicine is responsible for the first step of the binding mechanism, and the second step involves the rearrangement of the initial complex to interact with the A-ring. In this model, therefore, the initial and the second subsites are identified as the binding site for the C-ring and for the A-ring respectively.

Equilibrium binding studies of two- and three-ring analogues

The assumption of the existence of two-subsites seems to be confirmed by the fact that the analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTC), which lacks the B-ring (see Fig. 1), binds to tubulin with very high efficiency (Fitzgerald 1976). Equilibrium binding studies were performed by Andreu et al. (1984) and by Bane et al. (1984). The role of the B-ring and C-7 substitutions in the thermodynamics of colchicinoid tubulin interactions has been studied by Chakrabarti et al. (1996), and in the overall binding kinetics by Pyles and Bane (1993). It is generally accepted that the presence of the B-ring gives rigidity to the colchicine molecule. Relative to the parent molecule the binding of the two-ring analogues therefore results in a smaller negative free energy of binding, owing to the concomitant loss of rotational entropy (Medrano et al. 1989, Menéndez et al. 1989), and the absence of the B-ring speeds up the binding process considerably.

An interesting analogue of colchicine with a minor modification of the C-ring is colchicide (IDE) (see Fig. 1). In IDE the C-10 methoxy group of ring C is replaced by a hydrogen atom. It is reported to be a potent inhibitor of [^{3}]COL binding to tubulin and an inhibitor of microtubule assembly (Brossi et al. 1983; Staretz and Bane 1993). Isocolchicine (ISO) (Fig. 1) is a structural isomer of colchicine that differs in the relative positions of the methoxy and the carbonyl moieties of the C-ring. Its binding to tubulin was studied by Bane Hastie et al. (1989).

Kinetic studies of the two-ring analogue MTC

Assuming the existence of two-subsites, the kinetic behaviour of MTC is expected to be totally analogous to colchicine. It was therefore very surprising to find that the ki-

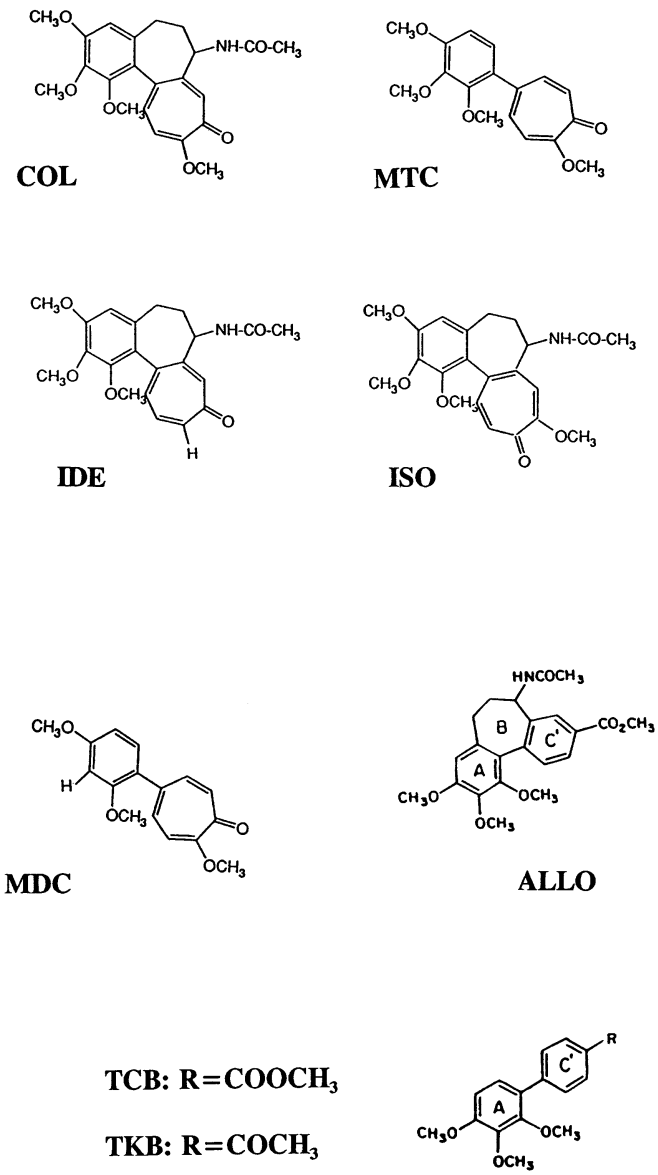

Fig. 1 The structures of the different analogs of colchicine that are referred to in the text

netic pathway of MTC binding to tubulin turned out to be very different from that of colchicine (Engelborghs and Fitzgerald 1986, 1987). In order to reconcile the assumption of the two subsites with the aberrant behaviour of MTC, it was proposed that MTC could enter the binding site in two ways: the classical one with the C-ring first and an alternative one in which the A-ring binds first (Andreu et al. 1991).

Kinetic studies of single ring analogues of colchicine

To test if the single ring analogues bind to the overall binding site of colchicine, kinetic studies using competition with MTC binding were performed. Since most single ring competitors do not give an optical signal, the fluorescence increase of the fast binding analogue MTC may be used as the probe signal. Three types of competitors can be expected a priori:

Class I: the competitor equilibrates much faster than MTC but binds with very low affinity.

$$\begin{matrix} T + MTC & \overset{K_{1,MTC}}{\rightleftarrows} & T \cdot MTC & \underset{k_{-2,MTC}}{\overset{k_{2,MTC}}{\rightleftarrows}} & T \cdot MTC^* \\ + & & & & \\ I & \underset{K_I}{\rightleftarrows} & T \cdot I & & \end{matrix} \quad (3)$$

with $K_{1,MTC}$ the initial binding constant of MTC, $k_{-2,MTC}$ and $k_{2,MTC}$ the rate constants for the second step in the binding process of MTC. The competitor is represented by I and K_I is its binding constant.

When tubulin is preequilibrated with such a fast, low affinity competitor, the competitor will slow down MTC binding proportionally to the saturation of the site, but will only marginally reduce the amplitude of the binding signal. (It might even be possible to discriminate the effect of competition on the first and on the second step, by determining the effect on the appropriate rate constants). For this type of competitor the following rate equation can be described:

$$\frac{d[T \cdot MTC^*]}{dt} = K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] \cdot \left[\frac{1}{1 + K_I \cdot [I]}\right] \cdot [T_0 - T \cdot MTC^*] - k_{-2,MTC} \cdot [T \cdot MTC^*] \quad (4)$$

leading to the following expression for the observed rate constant (the coefficient of $[T \cdot MTC^*]$):

$$k_{obs} = k_{-2,MTC} + \frac{K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC]}{1 + K_I \cdot [I]} \quad (5)$$

Note that the binding is studied at low MTC concentrations.

Class II: the competitor binds with rate constants similar to MTC, at comparable concentrations. When a mixture of the competitor, at increasing concentrations, and MTC is added to tubulin, two phases will be observed. In the first phase the ratio of complex formation by the two drugs will be determined only by the ratio of the two on-rates (and not by the equilibrium constants).

$$\begin{matrix} & + MTC & \overset{K_{1,MTC}}{\rightarrow} & T \cdot MTC^* & \overset{k_{2,MTC}}{} \\ T & & & & \\ & + I & \underset{k_I}{\rightarrow} & T \cdot I & \end{matrix} \quad (6)$$

Since only MTC is contributing to the signal, the amplitude will decrease with increasing competitor. The apparent rate constant of MTC binding in this first phase will, however, increase due to a faster approach to the (tempo-

ral) end state. In a second phase the ratio of the occupation by the two drugs will shift towards the true equilibrium situation by displacement of the weaker binding drug by the stronger binding one. Podophyllotoxin is such a clearcut case (Engelborghs and Fitzgerald 1987). In conditions where the dissociation rate constants can be neglected (first phase) the equations for parallel reactions (Capellos and Bielski 1972) can be applied, leading to the following equations:

$$\frac{d[T]}{dt} = -\left(K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] + k_I \cdot [I]\right) \cdot [T] \quad (7)$$

which can be integrated:

$$[T] = [T_0] \cdot \exp\left(-\left(K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] + k_I \cdot [I]\right) \cdot t\right) \quad (8)$$

furthermore

$$\frac{d[T \cdot MTC^*]}{dt} = K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] \cdot [T] \quad (9)$$

substitution followed by integration gives

$$[T \cdot MTC^*] = [T_0] \cdot \frac{K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC]}{K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] + k_I \cdot [I]} \cdot \exp\left(-\left(K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] + k_I \cdot [I]\right)\right) \quad (10)$$

with k_I being the bimolecular rate constant for the binding of I. The amplitude of the reaction is thus reduced by the factor:

$$\text{amplitude factor} = \frac{K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC]}{K_{1,MTC} \cdot k_{2,MTC} [MTC] + k_I \cdot [I]} \quad (11)$$

Class III: the competitor binds and dissociates very slowly. (Same scheme as (3)). Preincubation of tubulin with the competitor and subsequent study of the binding of MTC will lead to the loss of binding sites (amplitude) for MTC, while the kinetics of binding of MTC at the remaining open sites will not be influenced. In a second very slow phase the competitor will eventually be displaced. The observed rate constant of the first phase does not change when compared to the case in the absence of competitor and follows the simple relation (at low MTC concentrations) with reduced amplitude:

$$\frac{d[T \cdot MTC^*]}{dt} = K_1 \cdot k_2 \cdot [MTC] \cdot [T_0 - T \cdot MTC^* - T \cdot I] - k_{-2,MTC} \cdot [T \cdot MTC^*] \quad (12)$$

with $[T \cdot I]$ being a constant concentration in the first phase, owing to the slow exchange. The observed rate constant is:

$$k_{obs} = k_{-2,MTC} + K_{1,MTC} \cdot k_{2,MTC} \cdot [MTC] \quad (13)$$

TME behaves as a class III competitor: its binding does not give an optical signal, but it was visualized by the loss of free colchicine binding sites studied as a function of time. Its binding is extremely slow and is described by a bimolecular rate constant of $4.5 \pm 0.3 \times 10^{-2}$ $M^{-1}s^{-1}$ at 25 °C and an activation energy of 37 ± 8 kJ · mol^{-1}. By displacement experiments at 25 °C a dissociation rate constant of $4.8 \pm 1 \times 10^{-4}$ s^{-1} was determined. The equilibrium binding constant obtained was 60 ± 7 M^{-1} from equilibrium competition with colchicine and 90 ± 11 M^{-1} from kinetic measurements (Engelborghs et al. 1993). Its binding is therefore much slower than colchicine itself. Since TME has no influence on the kinetics of colchicine binding (apart from reducing the extent of binding after long incubation) a fast binding in competition with colchicine can be excluded. This behaviour is difficult to understand if the C-ring is responsible for the initial binding. On the contrary, the kinetic behaviour of TME seems to mimick the second step of colchicine binding. The fact that colchicine binds even faster than TME can be explained on the basis of a local high concentration effect after initial binding of the A-ring to a low affinity site.

Competition studies between NAM and MTC do not show an appreciable effect on the rate constants or on the amplitude of MTC binding (Dumortier and Engelborghs, unpublished results). However, a pronounced effect of NAM is observed with the colchicine analogue MDC, where one methoxy group in the A-ring is replaced by a hydrogen. The amplitude of the binding signal of this molecule is strongly reduced by NAM (Dumortier et al. 1997b). The simplest interpretation would be competition for the same sites. However, binding of NAM (present in excess) to the tubulin-MDC complex occurs with a rate constant different from the off-rate constant of MDC. The interpretation was therefore that NAM binds in the neighbourhood of MDC and quenches its fluorescence. From the quantitative analysis of this effect a binding constant for NAM of 320 ± 30 M^{-1} could be deduced. The NAM binding site that is seen by MDC is not seen by MTC or does not exist on the tubulin-MTC complex. These results also suggest a (probably limited) difference in the mode of binding of MTC and MDC, despite the very small difference to structure.

An example of fast competition with colchicine on the other hand is observed with a potential A-analogue TMA (3′,4′,5′-trimethoxyacetophenone) (Engelborghs et al. 1993). It reduces the rate constant of MTC binding when it is added to tubulin together with MTC. However, in addition to a fast effect it also seems to have an effect on longer time scales, probably indicating a second binding site. This is again confirmed with MDC, where a slow signal of TMA binding is observed after preincubation of tubulin with MDC (Dumortier et al. 1997b). It is interesting to note that isocolchicine behaves in a similar way to TMA: it competes very rapidly with colchicine binding, yet shows evidence for a second slow reacting binding site that can be visualized in the presence of the molecule MDL (MDL 27048 or trans-1-(2,5 dimethoxyphenyl)-3-[4-{dimethylamino)phenyl]-2-methyl-2-propen-1-one) (Dumortier et al. 1997a).

The results obtained with the single ring analogous of colchicine are puzzling, but at least clearly show that there must be more than one binding site for them on tubulin, at least one binding site that allows fast exchange, and one with much slower exchange kinetics.

Opposite effects on first and second steps

Among the many three-ring analogues of colchicine, a set of four are very interesting because they allow the construction of a thermodynamic pathway analogous to the double mutant cycle which is routinely applied to the study of protein-protein interactions (Schreiber and Fersht 1995). In this field the contribution of a side chain to the overall stability of a protein-protein complex, is quantitatively determined by measuring the difference in free energy of binding ($\Delta\Delta G^\circ_{binding}$) of the wild-type and the mutant protein to the same target protein. Another mutant protein can be made to study the contribution of a second residue. To find out if the two residues contribute independently, or in a cooperative way, the double mutant protein can be made and its binding studied. When $\Delta\Delta G^\circ_{binding}$ of the double mutant protein equals the sum of the $\Delta\Delta G^\circ_{binding}$ values of the two single mutant proteins, both residues contribute in an independent way to stability. When the $\Delta\Delta G^\circ_{binding}$ of the double mutant protein is smaller than the sum of $\Delta\Delta G^\circ_{binding}$ of the single mutant proteins, both residues contribute in a collaborative way i.e. the removal of the first residue also reduces the contribution of the second one to the overall stability of the complex.

The four molecules to which analogous reasoning can be applied are colchicine, MTC, allocolchicine (ALLO) and TCB (2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl). Compared to colchicine, MTC has lost the B-ring, in ALLO the C-ring is replaced by a modified phenyl ring, while in TCB both modifications are present together (Fig. 1). Here we concentrate on ΔH° values (since they show a much bigger variation than ΔG° values). When the change in $\Delta\Delta H^\circ_{overall}$ due to these two modifications is compared, full additivity is observed. However, when the thermodynamic schemes for the two steps are considered separately, deviations from additivity of ΔH°_1 and of ΔH°_2 are observed (Dumortier et al. 1996 a). The data here show that modifications of the C-ring and removal of the B-ring give results that influence each other. The data can be explained in two ways: (i) either the structure of the intermediate is assumed tot be the same for the four compounds and non-additivity of ΔH° points to cooperativity between the original groups for binding, or (ii) non-additivity of modifications follows directly from a different structure of the intermediate state. The latter is most likely in view of the fact that for overall binding additively of $\Delta H^\circ_{overall}$ is observed. Furthermore, the results clearly show that a modification that causes an increase in ΔH°_1, causes a decrease in ΔH°_2. Changing the C-ring into a six-membered ring causes a big increase in the enthalpy level of the final state, but also in the enthalpy level of the initial complex and the transition state. The biggest effect of removing the B-ring is the increase of the enthalpy level of the intermediate.

The compensating effect between the first and the second step is also true for many other compounds as can be seen in Fig. 2 and Fig. 3. When the ΔH°_1 for binding is plotted vs. ΔS°_1 a linear so-called “entropy-enthalpy compensation” plot is found. Since all the ΔG°_1 values for binding in the first step are within the range –21 ± 8 kJ/mol this is not surprising. Indeed one can rearrange the Van’Hoff equation into the following equation:

$$\Delta H^\circ_1 = -21(\pm 8) + T \cdot \Delta S^\circ_1 \qquad (14)$$

It is therefore not the linearity of the plot that is surprising, but the wide spread of values that are found for ΔH°_1

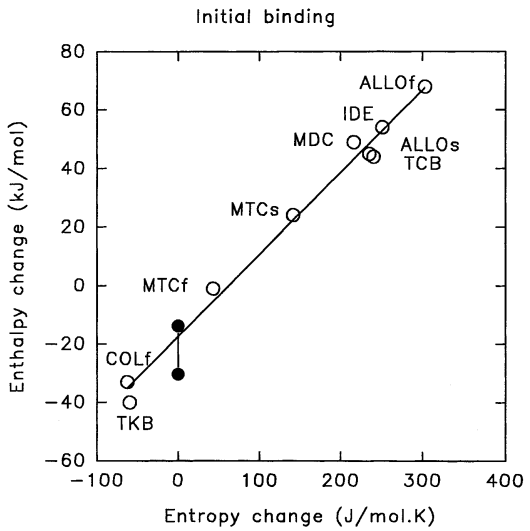

Fig. 2 The enthalpy change (kJ · mol^{-1}) for the initial binding is plotted vs. the corresponding entropy change (J · K^{-1} · mol^{-1}). The black circles refer to the range of free energy changes involved (ΔG°_1 varies between –28.8 and –13.3 kJ · mol^{-1}. Indications f and s refer to data for drug binding to the fast- and slow-binding tubulin isoform family respectively. For clarity error bars are omitted, they can be found in Table 1

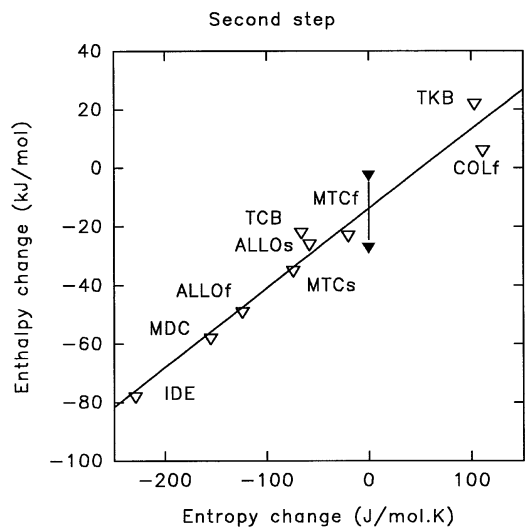

Fig. 3 The enthalpy change (kJ · mol^{-1}) for the second step of binding is plotted vs. the corresponding entropy change (J · K^{-1} · mol^{-1}). The black triangles refer to the range of free energy changes involved (ΔG°_2 varies between –27 and –2.4 kJ · mol^{-1}). Indications f and s refer to data for drug binding the fast- and slow-binding tubulin isoforms respectively. The values of the errors can be found in Table 1

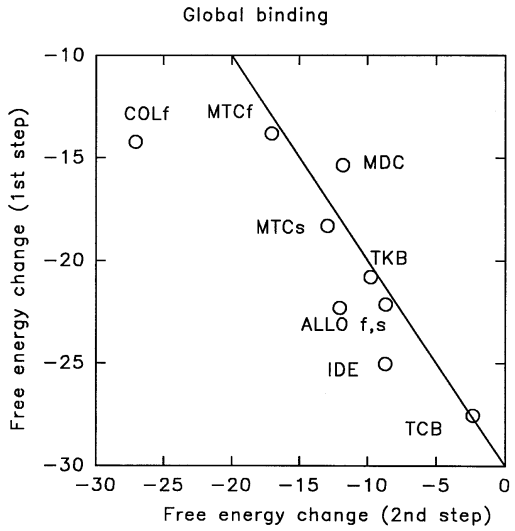

Fig. 4 Opposing effects of structural alterations as revealed by the changes in the free energy of binding at 25 °C for the initial step and the second step. The indications f and s refer to data for drug binding to the fast- and slow-binding tubulin isoforms respectively. For the sake of clarity error bars are omitted. The values of the errors can be found in Table 1

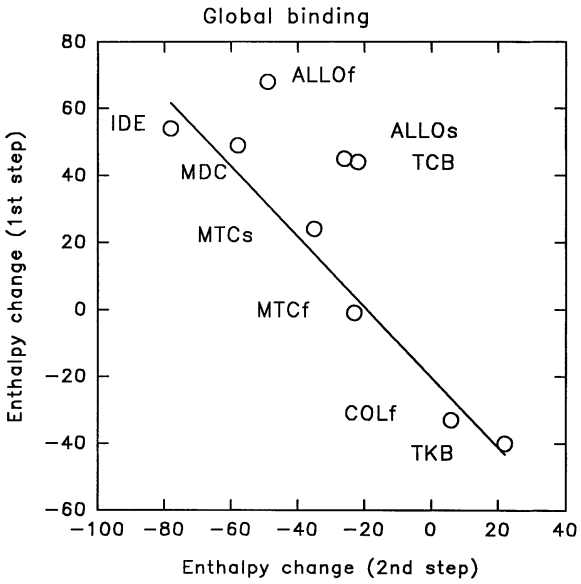

Fig. 5 Opposing effects of structural alterations as revealed by the changes in the enthalpy change of binding for the initial step and second step. The indications f and s refer to data for drug binding to the fast- and slow-binding tubulin isoforms respectively. The values of the errors can be found in Table 1

and ΔS_1° for the broad set of colchicine analogues, due to compensation between entropy and enthalpy changes (The same is also true for the second step, here ΔG_2° varies between 15 ± 12 kJ · mol^{-1}, see Fig. 3). The sequence of the products along the line in the graph is inverted in the second plot, again indicating opposing effects of structural alterations in the first as compared to the second step. It should be noted that molecules that compete with colchicine for the same site, but have very little or no structural resemblance also follow the same compensation plot (Silence et al. 1992; Barbier et al. 1996).

The plot of ΔG_1° vs. ΔG_2° is very revealing (Fig. 4). In a model of two independent subsites, it should be possible to change the ΔG° of binding of the two subsites independently. Therefore all data points should lie on an horizontal or a vertical line in the plot of Fig. 4. Most experimental data, however, follow a diagonal, indicating that the initial binding is increasing its stability at the expence of the second step. It is as if colchicine was designed to get the lowest affinity in the first step. Only the ΔG_1° of MTC binding to the fast-binding tubulin isoform is similar to the one of colchicine and lies on a horizontal line, indicating that the removal of the B-ring does not alter the free energy of the first step (despite a big change in the enthalpy) but decreases the equilibrium constant of the second step, a phenomenon that is observed for all other compounds.

The plot of ΔH_1° vs. ΔH_2° (Fig. 5) gives a similar diagonal relation and shows that all modifications (except TKB) lead to an increase of ΔH_1° (becomes endothermic) and a decrease of ΔH_2° (becomes exothermic). An intriguing question that arises is whether the conformationally linked effects (e.g. GTP-ase activity, changes in CD spectra, stability of the $\alpha\beta$-dimer) are uniquely linked to the second step or to overall binding. This question can only be answered by studying the kinetics of the appearance of the conformationally linked effects, which has not been done.

The fact that the enthalpies of most analogues lie on a diagonal in this ΔH_1° vs. ΔH_2° plot, means that a large part of the modifications of the thermodynamic parameters is compensated between the first and the second step. This is certainly true for the analogues lacking the B-ring. This compensation is, however, strongly violated in ALLO and TCB that both have a very different C-ring. Remarkably the extra modification of the C-ring in TKB as compared to TCB very strongly compensates for this effect.

Another remarkable effect is that the data for the slow and fast isoforms (f and s in Figs. 2–5) of tubulin behave in the same way. This is not surprising in the enthalpy/entropy plot, but it is surprising for the comparison of the parameters of the two sites. Yet the data for the isoforms can be very different for a given drug. It is clear that a careful study of the structure of the tubulin-colchicine complex, which is becoming available (Downing and Nogales 1998) will be able to explain some of these intriguing observations, including the fact that some drugs do not make a distinction between the fast and slow isoforms of tubulin.

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