Mechanism of tubulin–colchicine recognition: a kinetic study of the binding of the colchicine analogues colchicide and isocolchicine

Chantal DUMORTIER*, Qunying YAN†, Susan BANE† and Yves ENGELBORGHs*¹

*Laboratory of Chemical and Biological Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200D, B-3001 Leuven, Belgium, and †Department of Chemistry, State University of New York at Binghamton, Binghamton, NY 13902-6016, U.S.A.

Colchicide (IDE) is a colchicine (COL) analogue in which the C-10 methoxy group is replaced by a hydrogen atom. Its binding to tubulin is accompanied by a quenching of the protein fluorescence. The fluorescence decrease shows a monoexponential time dependence. The observed rate constant increases in a non-linear way with the total concentration of IDE, allowing the determination of a binding constant for an initial binding site ($K_1 = 5300 \pm 300 \text{ M}^{-1}$) and the rate constant for the subsequent isomerization ($k_2 = 0.071 \pm 0.002 \text{ s}^{-1}$) at 25 °C. The rate constant, k_{-2} , for the reversed isomerization can be determined by displacement experiments. Despite the minor alteration of the C-ring substituent, the kinetic and thermodynamic parameters of binding are substantially different from those of COL itself, for

both steps. In isocolchicine (ISO) the carbonyl oxygen atom and the methoxy groups of the C-ring have been interchanged. Its binding to tubulin only results in small fluorescence and absorbance changes. Therefore competition experiments with MTC [2-methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one] were performed. ISO competes rapidly and with low affinity with MTC. Fluorimetric titrations of tubulin with MDL (MDL 27048 or *trans*-1-(2,5 dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one) in the presence and absence of ISO give evidence for the existence of a second, slow-reacting low-affinity site for ISO that is not accessible to MTC or MDL. The relevance of these results for the recognition of COL is analysed.

INTRODUCTION

The binding of the alkaloid colchicine (COL; Figure 1) to tubulin occurs in two steps: a fast binding of low affinity, followed by a slow conformational change of the initial complex [1–3]. Different suggestions have been made to answer the question of what parts of the molecule are responsible for the initial and final interactions [4–7]. Therefore a kinetic study of analogues of COL with a small modification of the substituents on the C-ring was performed.

Colchicide (IDE) and isocolchicine (ISO; Figure 1) are compounds very similar in overall structure to COL. In IDE the C-10 methoxy group of ring C is replaced by a hydrogen atom. It was reported to be a potent inhibitor of [³H]COL binding to tubulin [8,9]. Its *in vitro* inhibition of microtubule assembly has been shown [9], but a quantitative kinetic study has not been performed. ISO is a structural isomer of COL that differs in the relative position of the methoxy and carbonyl moieties. Bane Hastie et al. [10] demonstrated that ISO competitively inhibits COL binding to tubulin and the assembly of tubulin into microtubules, but the affinity of this ligand for tubulin is much lower. We can imagine two binding sites for ISO: one for the intact A-ring not allowing the modified C-ring to enter its site and another site where the molecule is rotated and binds the C-subsite, but the A-ring cannot enter its normal subsite. However, the difference spectrum obtained upon binding shows peaks in the absorption region of the A-ring as well as the C-ring [10,11], indicating that both rings contribute to the low-affinity binding.

In order to obtain more details of the mechanism of binding of IDE and ISO, we have undertaken a detailed kinetic study of these compounds.

MATERIALS AND METHODS

Tubulin was prepared from pig brains as described previously [7,12]. This preparation contains the natural mixture of isoforms.

IDE was prepared by one of us (Q.Y.) from 10-demethoxy-10-fluorocolchicine by reaction with sodium borohydride as described by Staretz and Bane Hastie [9]. Stock solutions were made in a Mes buffer (50 mM Mes/70 mM NaCl/1 mM MgCl₂/1 mM EGTA/1 mM NaN₃, adjusted to pH 6.4 with NaOH). The IDE concentration was determined using a molar

Figure 1 Structure of colchicine and derivatives

Abbreviations used: COL, colchicine; IDE, colchicide; ISO, isocolchicine; MDL, MDL 27048 or *trans*-1-(2,5 dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one; MTC, 2-methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; TME, tropolone methyl ether.

¹ To whom correspondence should be addressed.

absorption coefficient of $8530 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 336 nm [9]. ISO was prepared by a semisynthesis from COL as described by Chapman et al. [13] and purified as described by Bane Hastie et al. [10]. It was dissolved in Mes buffer. Its concentration was measured with a molar absorption coefficient of $16270 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 348 nm [10]. 2-Methoxy-5-(2',3',4'-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) was prepared and purified by Dr. T. J. Fitzgerald as described previously [14]. Stock solutions were made by dissolving MTC in DMSO, and a molar absorption coefficient of $18.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 350 nm was used [15]. In the competition experiments with ISO, the final DMSO concentration was 2.5% (v/v) after dilution in the stopped flow.

trans-1-(2,5-Dimethoxyphenyl)-3-[4-(dimethylamino)phenyl]-2-methyl-2-propen-1-one (MDL) was a gift from Merrell Dow Laboratory, and its concentration was measured using a molar absorption coefficient of $21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 398 nm [16]. This molecule was dissolved in DMSO and diluted in Mes buffer that was adjusted to a final concentration of 2.5% DMSO.

All drugs were chromatographically pure.

Ligand binding kinetics

All kinetic studies were done with pure tubulin in Mes buffer at pH 6.4, ionic strength 0.1 M; 1 mM GDP was added to prevent polymerization.

The association kinetics of IDE were assessed by monitoring quenching of intrinsic protein fluorescence using a SPEX spectrofluorimeter. The excitation monochromator was set at 280 nm and the emission monochromator at 335 nm. The slit widths were 2 mm, with a resolution of 7.2 nm for excitation and 3.6 nm for emission.

The kinetic competition experiments with ISO and the dissociation kinetics of IDE were measured by stop-flow fluorescence (as described in [7]). Excitation was at 370 nm and the emission was collected through a Kodak Wratten filter 2B (cut-off at 395 nm). The dead time was 1.5 ms, according to [17]. The data obtained were fitted using non-linear least-squares curve-fitting software based on the Marquardt algorithm [18]. All other fittings were done with the program Sigmaplot®.

Fluorimetric titrations

The association constants for the binding of MDL to tubulin in the absence and presence of ISO were determined using the SPEX spectrofluorimeter. The temperature was controlled at 25 °C using a circulating-water bath. The excitation wavelength (420 nm) was chosen to minimize the inner filter effect and the emission wavelength was 485 nm. Different concentrations of MDL were added to the tubulin solution (1.9 μM preincubated with ISO or not) and the fluorescence intensities were determined after completion of the reaction. The emission intensity of MDL in the absence of tubulin was measured at different concentrations and these values, which were practically negligible, were subtracted. The data were analysed according to the following equation, which can be derived from the law of mass action:

$$KF = F_0 + aC_0 + (b - a) \cdot \frac{(T_0 + C_0 + K_d') - \sqrt{(T_0 + C_0 + K_d')^2 - 4T_0C_0}}{2} \quad (1)$$

where F_0 is the emission intensity of the protein without MDL, a and b are the intrinsic fluorescence of free and bound MDL respectively, T_0 is the initial tubulin concentration, C_0 is the total MDL concentration corrected for dilution and K_d' is the apparent dissociation constant for the MDL-tubulin complex in the presence of ISO.

In the case of fast competition, the dissociation constant of ISO can be determined from its competition equation:

$$K_d' = K_d(1 + [\text{ISO}]/K_{d,\text{iso}}) \quad (2)$$

where K_d' and K_d are the apparent dissociation constants for the binding of MDL to tubulin in the presence and absence of ISO respectively, and $K_{d,\text{iso}}$ is the dissociation constant of the tubulin-ISO complex.

RESULTS

Association kinetics of IDE

IDE does not show an increased fluorescence when bound to tubulin. However, like COL and MTC, IDE quenches intrinsic protein fluorescence upon binding to tubulin [5,15]. The decrease in tubulin fluorescence as a function of time was therefore monitored to evaluate the kinetics of the association. Good-quality curves were obtained with a signal-to-noise ratio of 15/1 (results not shown). The data could be fitted with a single exponential. The observed rate constants vary in a non-linear way with the drug concentration, indicating that the binding of IDE follows the well-known two-step mechanism of COL, MTC and other analogues [1,7,19–22]. The rate constant for the two-step mechanism under pseudo-first-order conditions is given by the following hyperbolic expression:

$$k_{\text{obs}} = k_{-2} + [k_2 \cdot K_1 \cdot [\text{IDE}]/(1 + K_1 \cdot [\text{IDE}])] \quad (3)$$

where K_1 is the equilibrium constant for the initial binding and k_2 and k_{-2} are the forward and backward rate constants for the isomerization of the initial complex ($K_2 = k_2/k_{-2}$); k_{-2} is the value obtained from displacement experiments.

The temperature dependence allows the calculation of the thermodynamic parameters for the initial binding and the activation energy for the isomerization (Table 1).

Dissociation kinetics of IDE

The dissociation process of IDE from its complex was studied by displacement experiments in the presence of a large excess of MTC. Tubulin (5 μM) was preincubated with IDE (10 μM) for 15 min and then MTC (200 μM) was added to the solution. Every binding site that becomes free upon dissociation should immediately be occupied by MTC (since $k_+[\text{MTC}] \gg k_+[\text{C}]$). In

Table 1 Kinetic and thermodynamic parameters of the individual steps of IDE and COL binding (fast phase)

Rate constants were taken at 25 °C.

	IDE	COL*
K_1 (M^{-1})	5300 ± 300	220
ΔH_1° ($\text{kJ} \cdot \text{mol}^{-1}$)	54 ± 7	-33 ± 12
ΔS_1° ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)	251 ± 23	-63 ± 40
k_2 (s^{-1})	0.071 ± 0.002	0.3
$E_{a,2}$ ($\text{kJ} \cdot \text{mol}^{-1}$)	26 ± 3	100 ± 5
$E_{a,\text{ass}}$ ($\text{kJ} \cdot \text{mol}^{-1}$)	80 ± 8	67 ± 13
k_{-2} (s^{-1})	$1.5(\pm 0.1) \times 10^{-3}$	$5.3 \times 10^{-6}\dagger$
K_2	47 ± 3	56603
$E_{a,-2}$ ($\text{kJ} \cdot \text{mol}^{-1}$)	104 ± 15	$94 \pm 10\dagger$
$\Delta H_{\text{tot}}^\circ$ ($\text{kJ} \cdot \text{mol}^{-1}$)	-24 ± 16	-27 ± 10
K_b (M^{-1})	$2.5(\pm 0.2) \times 10^5$	1.2×10^7

* Ref. [2].

† Ref. [3].

this experiment the reversed isomerization of tubulin–IDE (with rate constant k_{-2}) is the rate-limiting step for the binding of MTC. The rate constant has a value of $0.0015 \pm 0.0001 \text{ s}^{-1}$ at 25°C . These experiments were repeated at different temperatures and an activation energy of the reverse isomerization of $104 \pm 15 \text{ kJ} \cdot \text{mol}^{-1}$ was calculated.

Combining the data from the association and dissociation kinetics, the full pathway of complex formation can be described and compared with the previously determined pathway of COL (Table 1).

Interaction of ISO with tubulin

The tubulin–ISO complex is very weakly fluorescent compared with tubulin-bound COL [10]. The binding of ISO to tubulin could not be studied directly by stopped flow due to the low signal-to-noise ratio and the binding did not quench the intrinsic fluorescence of tubulin. Therefore the binding of ISO to tubulin was studied by kinetic competition experiments with MTC. In the stopped flow, tubulin was mixed with a solution containing both MTC ($15 \mu\text{M}$) and ISO (varying concentrations). Only MTC binding contributes to the amplitude of the fluorescent signal. Signal-to-noise ratios of 12:1 were obtained. Under these conditions of low tubulin and MTC concentrations, the experimental curves can be fitted to a single exponential. The amplitude of the MTC binding decreases in the presence of ISO, due to inner filter effects caused by ISO at 370 nm , and the observed rate constant decreases with increasing ISO concentrations. The decrease of the observed rate constant suggests that ISO competes very rapidly with MTC for its binding site.

From the decrease of the observed rate constant (presence versus absence of ISO), a binding curve could be plotted and a binding constant of $600 \pm 30 \text{ M}^{-1}$ at 24°C could be estimated, assuming a zero rate constant at saturation with ISO. (However, only 25% of the binding curve could be obtained.) The binding of ISO is essentially thermo-neutral, since the rate constant did not depend on temperature.

When tubulin was preincubated with ISO ($500 \mu\text{M}$) for different time scales (up to 1 h) and then the binding was studied, the amplitude of the fluorescent signal was not reduced relative to the reference. This indicates that there is no additional slow effect of ISO on MTC binding.

To study the mechanism of binding of ISO to tubulin in an independent way, the binding constant of ISO was determined by fluorimetric competition experiments. Because of the strong absorption of ISO at the absorption maximum of MTC, it was impossible to measure the binding of MTC fluorimetrically in the presence of ISO. However, MDL could be taken as the reference ligand, with an absorption maximum at approx. 400 nm [16]. To exclude the inner filter effects caused by ISO, the excitation was done at 420 nm . Fluorimetric titrations were performed as described in the Materials and methods section. The data are shown in Figure 2. The values of K_d for the MDL binding calculated from eqn. (1) were respectively $1.2 \pm 0.1 \mu\text{M}$ at $370 \mu\text{M}$ ISO, $1.2 \pm 0.1 \mu\text{M}$ at $740 \mu\text{M}$ ISO and $1.12 \pm 0.09 \mu\text{M}$ at $2960 \mu\text{M}$ ISO. In the absence of ISO, the calculated K_d value was $1.2 \pm 0.2 \mu\text{M}$. This proves that eqn. (2) is not valid and that there is no rapid competition. The amplitude of the fluorescence increase reduced as follows (see inset to Figure 2): $F = (F_0 + F_\infty \cdot K \cdot [\text{ISO}]) / (1 + K \cdot [\text{ISO}])$ with F_0 and F_∞ the maximum fluorescence values of MDL binding without, and saturated with, ISO, and K the association constant of the binding of ISO. From this decrease, we could calculate an association constant of $1300 \pm 600 \text{ M}^{-1}$. Three explanations are possible: (1) ISO binds

Figure 2 Fluorimetric titration of MDL binding to tubulin in the absence (solid symbols) and presence (open symbols) of ISO

Tubulin ($1.9 \mu\text{M}$) was preincubated for 20 min at 25°C with ISO ($0, 370 \mu\text{M}, 740 \mu\text{M}$ and $2960 \mu\text{M}$) and then MDL was added (excitation at 420 nm and emission at 485 nm). The fluorescence of different free MDL concentrations is practically negligible and is shown by open squares. The data were analysed according to eqn. (1) described in the Materials and methods section. The amplitude of the fluorescence increase was fitted to $F = (F_0 + F_\infty \cdot K \cdot [\text{ISO}]) / (1 + K \cdot [\text{ISO}])$ (see inset) (a.u., arbitrarily scaled units).

and dissociates slowly from its site and behaves very similarly to tropolone methyl ether (TME), a C-ring COL analogue [6], or (2) ISO and MDL bind at different sites with ISO reducing the fluorescence increase upon MDL binding, or (3) there are optical effects present. The last possibility can be excluded. To distinguish the two other possibilities, we have performed displacement experiments. In these experiments, tubulin ($2 \mu\text{M}$) was incubated with MDL ($4 \mu\text{M}$) to form the tubulin–MDL complex and then an excess of ISO (2 mM) was added. These experiments were performed in a spectrophotometer and the slit widths were 1 mm to reduce photobleaching of MDL. A fluorescence decrease was observed that could be fitted to a two-exponential function (results not shown). The observed rate constants at 25°C ($0.071 \pm 0.002 \text{ s}^{-1}$ for the fast phase and $0.0009 \pm 0.00004 \text{ s}^{-1}$ for the slow phase), are significantly different from the values found from displacement experiments of bound MDL by an excess of MTC, measured by Silence et al. [22]. Furthermore, the inset to Figure 2 shows that F_∞ does not extrapolate to zero, which strongly indicates that ISO and MDL do not compete for the same site, despite the fact that the observed rate constants showed no ISO concentration dependence. This indicates that conformational changes are being observed.

To answer the question of whether ISO also binds very rapidly to this site, we have performed kinetic competition experiments (same experiment as with MTC) in the stopped flow at 420 nm . When tubulin was mixed with a solution containing both MDL ($14 \mu\text{M}$) and ISO (0.5 or 1 mM), the amplitude and the observed rate constant of the MDL binding remained constant, as in the absence of ISO. But when tubulin was preincubated with ISO for 30 min and then the MDL binding was observed, the amplitude of the fluorescent signal decreased, whereas the observed rate constant did not change with increasing ISO concentrations.

From the decrease of the amplitude, a binding constant for ISO of $1050 \pm 100 \text{ M}^{-1}$ could be calculated. These results confirm that ISO binds slowly to a second site, which influences the fluorescence increase upon MDL binding, but not that of the MTC binding.

DISCUSSION

Kinetic analysis of the interaction of IDE with tubulin

Under pseudo-first-order conditions, IDE binding to tubulin displayed a single phase in the association reaction. It was previously shown that the kinetics of COL binding to tubulin are biphasic in both ligand fluorescence enhancement and tubulin fluorescence quenching under pseudo-first-order conditions, and that the global reaction parameters obtained from monitoring either signal are the same [23]. The two phases of the association reaction have been assigned to binding to tubulin isotypes [24–26]. We can therefore conclude that IDE is unable to distinguish tubulin isotypes kinetically.

The minor structural difference between COL and IDE leads to a dramatic difference in the details of the interaction of the two molecules with tubulin. Since the initial binding parameters are strongly different, these results suggest that ring C of IDE is involved in the initial binding. However, the high affinity of the initial binding step and the low affinity of the second step strongly suggest that a larger part of the molecule contributes to the initial binding than in the case of COL. Moreover, the pathway shows that in terms of standard enthalpy change, the intermediate is much more on the correct pathway to the transition state, than in the case of COL. It looks as if the C-10 methoxy group present in COL prevents, by steric hindrance, the binding in the initial site of IDE.

The kinetic parameters for the second step of the association of the two ligands with tubulin are also quite different. The activation energy of the isomerization is approx. $75 \text{ kJ} \cdot \text{mol}^{-1}$ less for IDE–tubulin than for COL–tubulin. Interestingly, the global activation energies of the two binding processes are the same within experimental error (Table 1). In fact, the global activation energies for the binding to tubulin of all tricyclic amide-containing colchicinoids so far investigated, i.e. COL, IDE, allocolchine [27], thiocolchicine [28], are in the range of 80 – $100 \text{ kJ} \cdot \text{mol}^{-1}$. From these results it is clear that the enthalpy position of the transition state and the final state are much less sensitive to the nature of the substituent on the C-ring than to the position of the intermediate.

Kinetic study of the binding of ISO to tubulin

Previous binding experiments using Hummel–Dreyer techniques gave a binding constant of $4.4 \times 10^3 \text{ M}^{-1}$ and, using competition with COL, gave $2.5 \times 10^3 \text{ M}^{-1}$ [10]. Our binding constant, obtained from competition with MTC, is much smaller (600 M^{-1}). The fact that we are only able to obtain 25% of the binding curve could be responsible for this low value. The experiments suggest that there are two types of low-affinity binding sites for ISO. One type of site overlaps part of the MTC binding site, but not the MDL binding site. ISO binds rapidly and with very low affinity to this site. We will refer to this site as the ‘first site’. The kinetic

behaviour of ISO for this site resembles the behaviour of A-ring analogues.

The ‘second site’ is situated close to the MDL site but does not overlap the MTC nor the MDL binding site. ISO binds considerably more slowly and with higher affinity to the second site than to the first. The possibility cannot be excluded that this binding site is created directly or indirectly by the binding of MDL.

Absorption difference spectroscopy of ISO in the presence and absence of tubulin shows perturbation of the tropone chromophore and the trimethoxyphenyl chromophore, indicating that the fast-binding mode of ISO involves interaction of both rings with the protein. This is in agreement with the initial binding of all COL derivatives studied so far.

The rapid binding of ISO in the first site, and the rapid binding of all COL analogues, is in sharp contrast with the behaviour of TME itself, which binds only very slowly [6]. These data suggest that the slow site detected for TME is not directly accessible to COL and derivatives, probably by (steric) hindrance from the rest of the molecule.

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