Response of microtubules to the addition of colchicine and tubulin–colchicine: evaluation of models for the interaction of drugs with microtubules

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The effects of free drug and tubulin–drug complexes on steady-state GTP/GDP-associated microtubules and on equilibrium guanosine 5′-[β,γ-imido]triphosphate-associated microtubules are compared. The addition of colchicine or the tubulin–colchicine complex (TuCol) to steady-state microtubules induces microtubule disassembly. Only limited disassembly of equilibrium microtubules is observed under similar conditions. Addition of colchicine or the bifunctional colchicine analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone to preassembled steady-state or equilibrium microtubules does induce disassembly, but establishment of the new steady state or equilibrium is very slow. These observations are related to the fact that TuCol readily adds to the microtubule end, but is only incorporated into the lattice with difficulty. As a result, microtubule growth is effectively inhibited and the critical concentration is significantly increased. Nevertheless, drug-induced disassembly can be extremely slow, because the frequency of addition reactions increases as the concentration of soluble dimers increases. The efficiency of incorporation of TuCol decreases as its concentration increases. This work further confirms the existence of colchicine-binding sites with low affinity (association constant $K_{MT} \sim 3 \times 10^2\ M^{-1}$) along the microtubule lattice. This value suggests that part of the colchicine-binding site on tubulin remains available in the polymer. The interaction of colchicine with these sites has no appreciable effect on microtubule dynamics. These observations are reproduced and rationalized by the model described elsewhere [Vandecandelaere, Martin, Bayley and Schilstra (1994) Biochemistry **33**, 2792–2801], and the possibility that there are co-operative effects in the inhibition is considered.

INTRODUCTION

Owing to its potent anti-mitotic properties, the alkaloid colchicine has been the subject of scientific interest for many years (for reviews, see [1,2]). Colchicine completely inhibits microtubule self-assembly at a level well below the concentration necessary to saturate the available tubulin, both *in vitro* [3] and *in vivo* [4]. This phenomenon is known as substoichiometric inhibition. On the other hand, large suprastoichiometric amounts of colchicine fail to induce complete disassembly of preformed microtubules [5] (for review, see [2]). These observations are not consistent with the drug perturbing a simple dimer/polymer balance, indicating that colchicine has complex effects on microtubule dynamics. Initial binding studies failed to demonstrate significant binding of colchicine to intact microtubules [6,7]. Consequently, all further research focused on the effects of the drug on the microtubule ends. The drug affects end dynamics through the prior formation of tubulin–colchicine complexes (TuCol) [8–10]. These complexes bind rapidly and reversibly to the microtubule ends, with an affinity ($K_d \sim 0.3\ \mu M$ at 35 °C) similar to that of unbound tubulin [9,11]. TuCol inhibits microtubule elongation [11–15], but does not seem to affect the nucleation process [11,16]. TuCol inhibits growth at both microtubule ends to a similar extent [13–15]. Under suitable conditions, relatively large amounts of TuCol are incorporated into the microtubule lattice [12,17,18], but the co-assembly of tubulin and TuCol into the microtubule is not a conventional co-polymerization reaction [17]. Conventional co-polymerization of tubulin and TuCol at high Mg^{2+} concentrations produces long ribbon-like structures, indicating that the thermodynamically favoured conformation of TuCol under these conditions cannot be accommodated in the normal microtubule lattice [19–22].

Attempts to model the effects of TuCol on microtubule dynamics have used two approaches. (1) Analytical models have been used to describe the system in terms of overall thermodynamic and kinetic parameters [9,16,17,23]. Such models consider the affinity of the drug for soluble tubulin, the strong reversible binding of TuCol at the microtubule ends and the finite likelihood of the incorporation of TuCol into the microtubule. Although the analytical approach has provided valuable information, its weakness is that the interaction of TuCol with the microtubule ends has to be greatly simplified in order to enable the appropriate differential equations to be solved. (2) Recently we have tried to circumvent this problem by describing the effects of TuCol [and, more generally, of any tubulin–drug complex (TuDrug)] on microtubule dynamics using the lateral cap model for microtubule dynamic instability [14]. The observed effects of TuCol on dynamic instability can be reproduced by numerical simulation if it is assumed that TuCol selectively inhibits growth along one direction of the helical lattice. The present work extends the application of this model by addressing the question of what happens when TuCol, colchicine or its

Abbreviations used: MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; tubulin-GTP, tubulin-GDP and tubulin-p[NH]ppG, tubulin dimer with GTP, GDP and p[NH]ppG respectively at the exchangeable nucleotide-binding site (E-site); PC-tubulin, tubulin free of microtubule-associated proteins; TuCol, tubulin–colchicine complex; TuDrug, tubulin–drug complex; PEMG′, microtubule assembly buffer; C_t, total concentration of tubulin in the system; C_s, concentration of tubulin in soluble phase; C_p, concentration of tubulin in the polymer phase; $[Tu]_s$, $[TuDrug]_s$ and $[TuCol]_s$, concentrations of free tubulin, TuDrug and TuCol in solution; J_{on}, overall microtubule growth rate.

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bifunctional analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone (MTC) is added to microtubules *in vitro*.

Microtubule ends owe their special dynamic properties to the hydrolysis of GTP [24], and their response to the addition of a drug may be related to these properties. Therefore the effects of drugs on steady-state GTP/GDP-associated microtubules and equilibrium guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG)-associated microtubules [25–27] have been compared. Special attention has also been given to the limited disassembly of microtubules in the presence of excess colchicine and MTC, and the incorporation of TuCol into the microtubules. Finally, since colchicine-binding sites along the microtubules have been reported [28,29], the affinity of the drug for these sites has been estimated in order to determine the extent to which a linked function relationship between the binding of colchicine to the microtubules and free dimer may interfere with the effects of TuCol on the dynamics of the microtubule ends. The results are rationalized using the relatively simple principles described previously [14], and a theoretical $J_{on}(C)$ plot [24] has been constructed (where J_{on} is the overall growth rate and C is the concentration of tubulin in solution). The $J_{on}(C)$ plot offers an understanding of the suprastoichiometric effect of colchicine and the substoichiometric inhibition of microtubule assembly.

MATERIALS AND METHODS

Microtubule protein was purified from pig brains by two cycles of temperature-dependent assembly and disassembly [30]. Tubulin free of microtubule-associated proteins (PC-tubulin) was isolated by chromatography on a Whatman P11 phosphocellulose column and subsequent gel filtration on Sephadex G-25. The absence of microtubule-associated proteins was verified by SDS/PAGE. Prior to each experiment the protein was subjected to a third cycle of assembly and disassembly, and resuspended in the appropriate buffer. All experiments were performed on PC-tubulin in 100 mM Pipes, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM NaN_3 and 3.4 M glycerol at pH 6.5 (PEMG′ buffer). Glycerol was added in order to increase the signal-to-noise ratio in the experiments by increasing both the polymer mass and the polymer number concentration to polymer mass ratio. Where appropriate, a GTP-regenerating enzyme system was added (1 unit/ml acetate kinase, 2.5 mM acetyl phosphate and 1.5 mM additional Mg^{2+}).

Colchicine was from Janssen Chimica, and MTC was a gift from Dr. T. J. Fitzgerald (College of Pharmacy, Florida Agricultural and Mechanical University, Tallahassee, FL, U.S.A.). GTP, p[NH]ppG, acetate kinase and acetyl phosphate were from Boehringer Mannheim. [^{3}H]Colchicine, [^{3}H]GTP and [^{14}C]GTP were from Amersham International. All other chemicals were of reagent grade. TuCol was prepared by mixing stoichiometric amounts of drug with protein and leaving the mixture for 90 min at room temperature.

Tubulin-p[NH]ppG (tubulin dimer with p[NH]ppG at the E-site) was prepared according to the method of Purich and McNeal [25] with the modifications described by Hinz and Timasheff [27]. PC-tubulin in PMG′ buffer (100 mM Pipes, 5 mM $MgCl_2$, 3.4 M glycerol, pH 6.5) was left at 37 °C for 50 min, and subsequently cooled again on ice for 30 min. All further operations were performed on ice. Alkaline phosphatase (4 units/mg of tubulin) and 0.03 mM $ZnSO_4$ were added and the solution was left for another 30 min. After the incubation, 1 mM EGTA and 5 mM p[NH]ppG were added and the solution was incubated for a further 2 h. Finally, the solution was clarified by centrifugation (37000 g; 4 °C) for 20 min. The resulting tubulin solution supported several cycles of temperature-dependent assembly and disassembly without additional GTP. Turbidity develops at room temperature and is reversible upon cooling to 4 °C. Cold-induced disassembly is slower than in a comparable sample of GTP/GDP-microtubules. The p[NH]ppG-microtubules cannot be distinguished from normal GTP/GDP-microtubules by electron microscopy (negative stain; results not shown). The critical concentration of the p[NH]ppG-microtubules at 37 °C was determined by consecutive dilution of preformed polymers, and found to be very low ($< 1\ \mu$M).

The response of steady-state microtubules upon addition of drug was monitored by measuring turbidity in a Varian Cary 118 spectrophotometer. The drug-dependence of the concentration of tubulin in solution (C_s) was examined by measuring the concentration of soluble protein in the supernatant after ultracentrifugation of the microtubule suspension. PC-tubulin (30–40 μM) was assembled in PEMG′ at 37 °C. Colchicine was added 30 min after the start of self-assembly, and the solutions were left to equilibrate for 1 h at 37 °C. After the incubation, the solutions were centrifuged (100000 g; 37 °C), and the concentration of tubulin in the supernatant was measured by the method of Bradford [30a]. Alternatively, the turbidity of the solutions was calibrated and, knowing the total concentration of tubulin in the system (C_t), the distribution of protein between the polymeric and soluble phases was calculated from turbidity changes. PC-tubulin (30 μM) was assembled in PEMG′ at 37 °C. At 60 min after the start of self-assembly, different amounts of MTC were added to the system, and the turbidity change of the solution was monitored at 400 nm. At 400 nm a turbidity change of 0.095 absorbance units/mg per ml of polymerized tubulin was recorded. The turbidity was monitored at 400 nm because of the high absorbance of the drug at 350 nm.

The incorporation of TuCol into microtubules was monitored using [^{3}H]TuCol (prepared by incubation of equimolar amounts of tubulin and labelled colchicine at room temperature for 90 min). Microtubules were assembled in PEMG′ in the presence of a GTP-regenerating system containing [^{14}C]GTP (15 μM [^{14}C]GTP, 1 unit/ml acetate kinase, 20 mM acetyl phosphate). At 30 min after the start of self-assembly, the solution was divided into aliquots and incubated with different amounts of [^{3}H]TuCol for 1 h at 37 °C. After the incubation, the aliquots (300 μl) were sedimented through 1 ml of PEM buffer containing 70 % (v/v) glycerol (2 h at 60000 g; 37 °C). The pellets were washed with warm (37 °C) PEMG′ and resuspended in 200 μl of ice-cold water. The relative amount of TuCol incorporated was determined from the ratio of ^{3}H radioactivity to ^{14}C radioactivity.

The affinity of colchicine for sites along the microtubule lattice was determined using p[NH]ppG-microtubules. Samples of tubulin-p[NH]ppG (55 μM) in PEMG′ were assembled by heating at 37 °C. [^{3}H]Colchicine was added 30 min after the start of self-assembly, and the samples were then left for 60 min at 37 °C. The microtubules were subsequently centrifuged (60000 g; 37 °C) through a 1 ml cushion of PEMG′ containing 70 % (v/v) glycerol. The supernatant was discarded and the pellets were washed three times with warm (37 °C) PEMG′ and then resuspended in ice-cold PEMG′. The tubulin concentration in the resuspended pellet was assessed by the method of Bradford [30a]. The concentration of colchicine in the pellet was determined by liquid scintillation counting. The degree of saturation of the tubulin, θ (= [[^{3}H]colchicine]/[tubulin]), was determined and the data were analysed using the equation:

$$\theta = \theta_{max} K_{MT}[\text{colchicine}]/(1 + K_{MT}[\text{colchicine}])$$

where θ_{max} is the number of lattice binding sites per tubulin dimer and K_{MT} is the association constant.

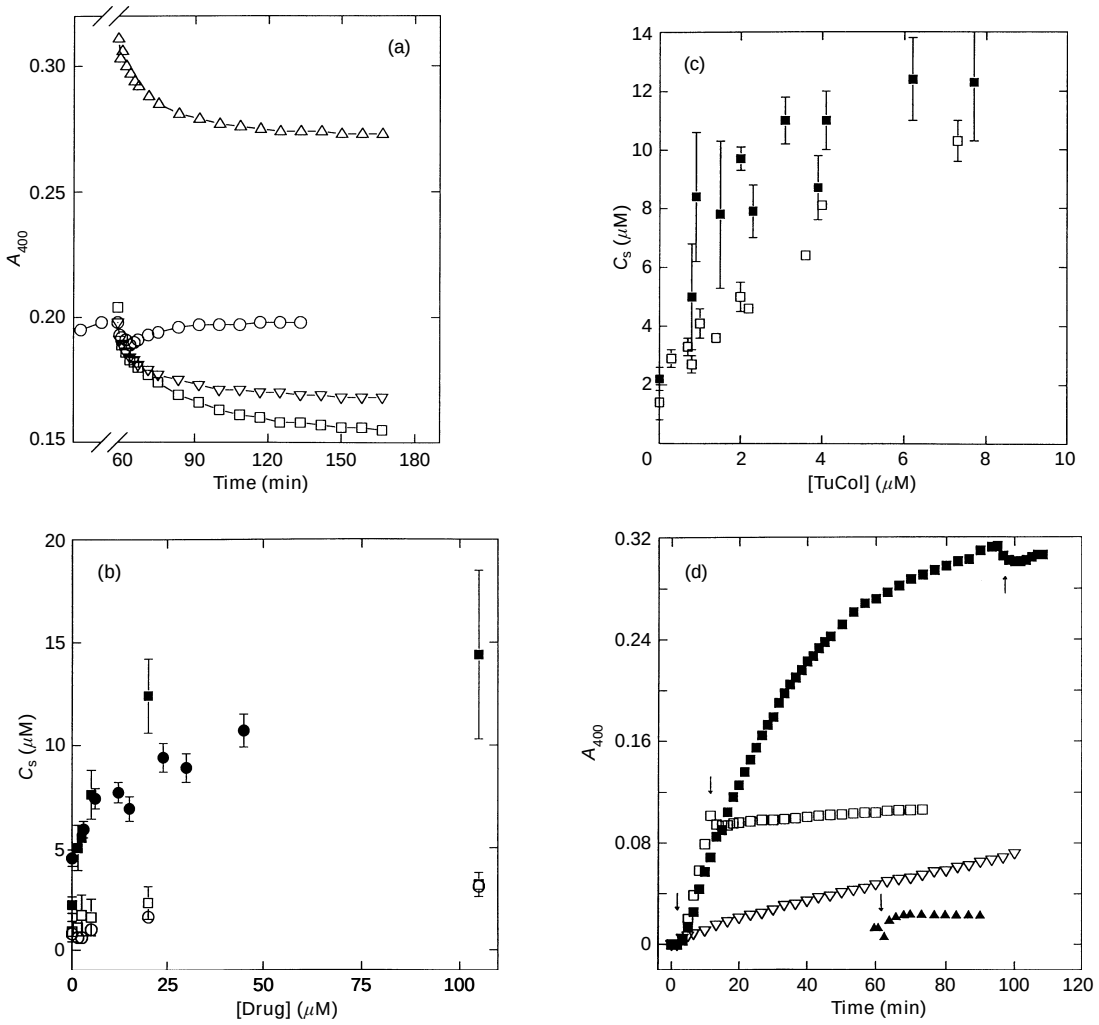

Figure 1 Response of microtubules to the addition of MTC, colchicine and TuCol

(**a**) PC-tubulin ($C_t = 25\ \mu$M) was assembled and the turbidity was monitored at $\lambda = 400$ nm. The drug was added 60 min after the start of assembly. Shown is the turbidity change after the addition of buffer (○), 3 μM MTC (▽), 30 μM MTC (□) and 185 μM MTC (▲). Note that some additional absorbance is introduced by the drug. (**b**) Concentration of tubulin in solution (C_s), 1 h after the addition of colchicine (■, □) or MTC (●, ○) to GTP/GDP-microtubules (■, ●) at steady-state, or to p[NH]ppG-microtubules (□, ○) at equilibrium. The effect of MTC on GTP/GDP-microtubules was derived from the turbidity change; in all other cases the tubulin in solution was separated from the microtubules by ultracentrifugation. (**c**) C_s 1 h after the addition of TuCol to GTP/GDP-microtubules (■) at steady-state, or to p[NH]ppG-microtubules (□) at equilibrium. (**d**) Effect of drug on the assembly of p[NH]ppG-microtubules: 25 μM MTC was mixed with 25 μM tubulin-p[NH]ppG after assembly (■), during elongation (□) and before self-assembly (▽). The last experiment was repeated with colchicine (▲); for clarity, this curve has been translated along the abscissa. The reaction was started by adding a suitable amount of cold tubulin-p[NH]ppG to warm buffer. The drug was added at the times indicated by the arrows, or was present in the buffer before the addition of tubulin-p[NH]ppG.

The response of single microtubules to the addition of TuDrug was simulated using the lateral cap model [31], with modifications to account for the effects of drugs. The calculations were performed on the β-out end of a 13-protofilament A-lattice (a typical site for dimer addition to the lattice is illustrated in Figure 4). The affinity of a dimer for the site (p16/z/p10) depends on the nature of the nucleotides in positions p16 and p10. It is assumed that the site-specific association rate constant, $k_{+,XY}$, is unaffected by the nature of the nucleotides on the neighbouring β-subunits; upon addition, any GTP in position z is hydrolysed (see [31] for details). The effect of TuDrug in an adjacent site in the lattice was simulated by introducing a decrease in $k_{+,XY}$ using $k_{+,XY,i} = k_{+,XY}/f$, where f is the inhibition factor. The inhibition can be in either the five-start or the eight-start helix direction [14]. In the present work, the inhibition was arbitrarily chosen to be in the five-start helix direction. Thus, in the simplest model, $k_{+,XY}$ for the addition on z was decreased 10- or 100-fold when position p16 contained the drug. The model was extended to account for possible co-operative effects in the inhibition (see the Discussion section): if only position p16 contains the drug, then $f = 10$; if both positions p16 and p6 contain the drug, then $f = 100$. In order to simulate the effects of TuDrug on the overall growth rate, J_{on}, of a single microtubule end, the total concentration of dimers in solution was kept constant. In order to simulate the dynamic response of a microtubule population to the addition of TuDrug, the total concentration of dimer in the system (polymer + dimer) was kept constant, and the polymer/dimer equilibrium was accounted for by introducing a microtubule number concentration of 5 nM. The simulation was performed for both GTP/GDP- and p[NH]ppG-microtubules. The known stabilizing effect of glycerol was accounted for by arbitrarily strengthening one lateral bond in the three-start helix direction

by 2.5 kJ/mol compared with the value used previously [31]. As a result, dynamic length excursions of the end are considerably decreased, the critical concentration is reduced from 8.25 μM to 1.5 μM and the rate constant for the dissociation of tubulin-GDP [tubulin dimer with GDP at the exchangeable nucleotide binding site (E-site)] from the tubulin-GDP lattice is 140 s^{-1} (cf. [24]). (Simulations using the unmodified model [31] show larger effects, but in qualitative terms the results are similar.) In order to reproduce equilibrium microtubules, the simulation program was modified by eliminating the GTP hydrolysis associated with dimer addition.

RESULTS

Effects of colchicine, TuCol and MTC on preassembled microtubules

The effects of MTC addition on preformed GTP/GDP-microtubules are illustrated in Figure 1(a). At all drug concentrations examined there was a rapid decrease in turbidity followed by a much slower change. Only partial disassembly was observed. Subsequent cooling to 4 °C resulted in rapid disassembly of the remaining polymer (results not shown; [9]), and no reassembly was observed when the solution was reheated to 37 °C. The increase in the concentration of dimers in solution, C_s (= $[Tu]_s + [TuDrug]_s = C_t - C_p$, where $[Tu]_s$ and $[TuDrug]_s$ are the concentrations of free tubulin and TuDrug in solution, and C_p is the concentration of tubulin in the polymer phase), is shown in Figure 1(b), which also shows the effects of colchicine on C_s for GTP/GDP-microtubules and p[NH]ppG-microtubules. In the case of GTP/GDP-microtubules, C_s increased from 2.5 μM in the absence of the drug to a plateau value of $\sim$ 13 μM at drug concentrations greater than 100 μM. This compares well with previous observations in PEM′ buffer with and without 1 M glycerol [14]. Although the absolute increase in C_s was significantly reduced at the high glycerol concentrations used here, the apparent plateau in C_s at high drug concentrations was always 4–5 times the value obtained in the absence of the drug. In this respect the response of p[NH]ppG-microtubules was similar, with C_s increasing from 0.9 ± 0.5 μM to 3.1 ± 0.7 μM in the presence of 105 μM colchicine.

The effects of TuCol on C_s are shown in Figure 1(c). The data for GTP/GDP-microtubules clearly show that the effect on C_s was not solely attributable to the amount of protein added as TuCol, i.e. the microtubules were destabilized by the TuCol. (In PEM buffer without glycerol, C_s is increased from 8 μM to 25 μM in the presence of 6 μM TuCol [14].) However, when TuCol was added to p[NH]ppG-microtubules the increase in C_s could be attributed to the protein added as TuCol, indicating that TuCol had little observable effect on p[NH]ppG-microtubules over the time scale of the measurements.

The importance of kinetic behaviour in the effects of drugs on microtubule assembly is illustrated in Figure 1(d). When a stoichiometric amount of MTC (25 μM) was added to p[NH]ppG-microtubules (C_t = 25 μM) during elongation or nucleation, immediate inhibition of further assembly was observed, indicating that the interaction of the drug with Tup[NH]ppG is rapid. A very small amount of assembly was observed when the protein was added to a similar amount of colchicine, after which the inhibition was complete. This difference reflects the slower, but irreversible, binding of colchicine to tubulin.

Negative-stain electron microscopy showed that microtubules were present in all the experiments described above, and that there was no apparent effect of the drugs on microtubule structure (results not shown).

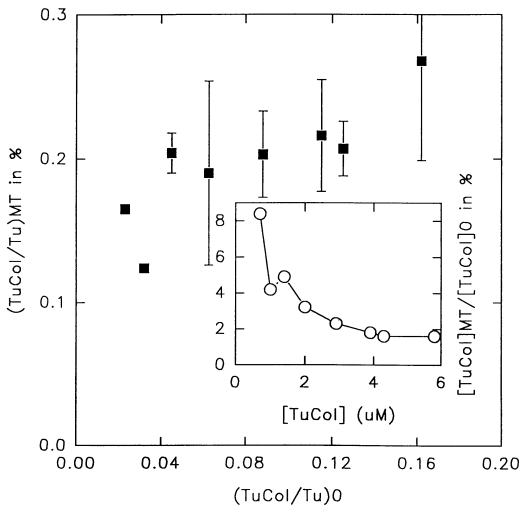

Figure 2 Incorporation of TuCol into the microtubule lattice

Microtubules at a steady state of assembly in PEMG′ were incubated at 37 °C for 1 h in the presence of radioactive TuCol, and subsequently centrifuged through a PEM′/glycerol cushion. Incorporation of the radioactive label into the pellet was measured. The relative incorporation {the TuCol/tubulin ratio in the pellet [(TuCol/Tu)MT]} is expressed as a function of the amount of TuCol in the system [(TuCol/Tu)O] (see the text). The insert shows the efficiency of incorporation, expressed as the amount of TuCol found in the pellet relative to the total amount of TuCol added to the system ([TuCol]MT/[TuCol]O).

Incorporation of TuCol into the microtubule lattice

The incorporation of TuCol into microtubules under the conditions described above was monitored. Figure 2 shows the relative incorporation of TuCol as a function of the fraction of the total tubulin present as TuCol. The insert shows the efficiency of incorporation expressed as the amount of TuCol incorporated relative to the total amount of TuCol added. The incorporation is clearly limited under these conditions. Between 0 and 5.8 μM TuCol was added to microtubules at steady state (C_t = 35 μM). Thus between 0 and 16 % of all tubulin in the system was TuCol. Taking the concentration of soluble tubulin as determined in Figure 1(b) into account, this means that up to 50 % of the soluble tubulin was present as TuCol. Yet, even under these conditions, only 0.27 ± 0.07 % of the polymer was labelled with the drug. At a Mg^{2+} concentration of 10 mM, the incorporation of TuCol into the polymer was one order of magnitude higher (results not shown). The percentage incorporation increased with increasing TuCol concentration to an apparent plateau of about 0.25 %. However, the efficiency of incorporation decreased with increasing TuCol concentration. Thus, when 0.7 μM TuCol was incubated with the microtubules, 8.4 % of it was incorporated after 60 min. In contrast, only 1.6 % of the TuCol became incorporated when its concentration was 5.8 μM. Combining the information from Figures 1(c) and 2, it can be shown that the concentration of the drug-free dimers in solution, $[Tu]_s$, is increased by the addition of TuCol.

Characterization of the drug-binding site on the microtubule lattice

Low-affinity binding sites for derivatives of colchicine exist on the microtubule lattice [28,29]. The greater stability of p[NH]ppG-microtubules allowed us to assess the affinity of colchicine for these lattice sites under our experimental con-

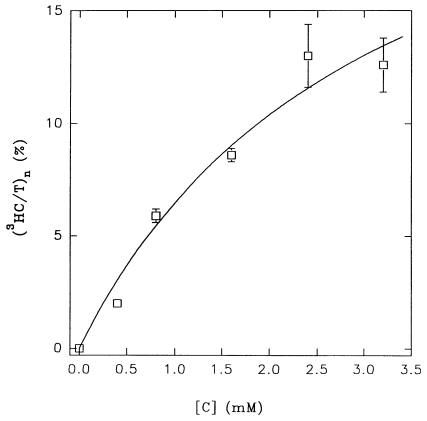

Figure 3 Interaction of [^{3}H]colchicine with p[NH]ppG-microtubules

Preassembled p[NH]ppG-microtubules were incubated with [^{3}H]colchicine for 1 h at 37 °C, and subsequently centrifuged through a PEM/glycerol cushion. Retention of the radioactive label in the pellet was measured, and results are plotted as the amount of colchicine per dimer in the pellet [$(^3HC/T)_n$] against the total amount of colchicine added ([C]).

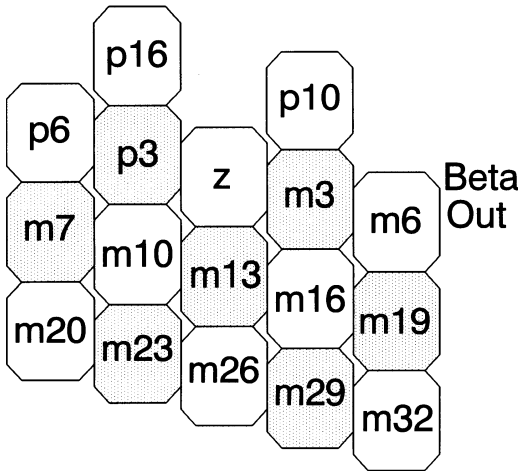

Figure 4 Inhibition by TuDrug: definition of models

A typical site (p16/z/p10) for the addition of a dimer to the β-out end of a 13-protofilament A-lattice is shown. In the lateral cap model, upon addition of a new dimer any GTP in the subunit in position z is hydrolysed. The presence of TuCol in the lattice affects the affinity for the adjacent dimer in either the five-start or the eight-start helix lattice direction. In order to model the effect of TuCol, the inhibition was arbitrarily chosen to be five-start helical. Thus, in the simplest model, the association rate constant, $k_{+,XY}$, for addition to the site (p16/z/p10) is decreased by a factor f, so that $k_{+,XY,i} = k_{+,XY}/f$ if the subunit in position p16 is affected by the drug. A more elaborate model illustrates the principle of co-operative effects in the inhibition, by assuming that $k_{+XY,i} = k_{+,XY}/f_1$ if only position p16 is affected by the drug, and $k_{+,XY,i} = k_{+,XY}/f_2$ (with $f_2 > f_1$) if both positions p16 and p6 are affected by the drug.

ditions (Figure 3). Values of the association constant ($K_{MT} = 325 \pm 163\ M^{-1}$) and of the stoichiometry ($\theta_{max} = 0.26 \pm 0.08$) were determined. The low affinity of colchicine for the lattice site presents an experimental problem: as microtubules migrate through the PEM/glycerol cushion during centrifugation (see the Materials and methods section), a substantial amount of the drug is likely to dissociate. This may explain why only 0.26 colchicine molecules per tubulin dimer were found, and the values of θ_{max} and K_{MT} are lower limits.

Simulation of the interference of TuCol with microtubule dynamics

The effects of TuCol on microtubule dynamics can be reproduced by computer simulations using procedures described elsewhere [14,31]. The effects of TuDrug are modelled by decreasing the association rate constant, $k_{+,XY}$, of the binding of an incoming dimer to the inhibited site adjacent to the TuDrug complex (Figure 4). The simple formulation used previously [14] can also be extended to incorporate more complex mechanisms of action; we considered the possibility of co-operativity in the inhibitory effect by an increased reduction of $k_{+,XY}$ for sites adjacent to two consecutive TuDrug complexes in the five-start lattice direction (see Figure 4). The overall rate of microtubule elongation (or disassembly), J_{on}, was calculated as a function of the total concentration of soluble tubulin, C_s ($= [Tu]_s + [TuDrug]_s$), and a $J_{on}(C)$ diagram was constructed (cf. [24]). From the $J_{on}(C)$ diagram, the effects of TuDrug on the critical concentration and the rates of microtubule growth and disassembly were derived. The simulations were designed to illustrate general features and principles rather than to reproduce in detail the experimental observations reported here.

Figure 5(a) shows the simulated effects of adding a specified amount of TuDrug on C_s and $[Tu]_s$. The presence of TuDrug in steady-state microtubules induces an additional disassembly of the microtubules, seen as an increase in $[Tu]_s$. By contrast, no such disassembly is observed when TuDrug is added to equilibrium microtubules, and the added TuDrug accumulates in the solution. The incorporation of TuDrug into steady-state microtubules is reproduced in Figure 5(b). In all cases the model predicts a reduced efficiency of TuDrug incorporation into the lattice. If the inhibition is weak (low f), a considerable amount of TuDrug is incorporated, whereas only low levels are incorporated when the inhibition is strong (high f).

Figure 5(c) shows the simulated response of a single microtubule end to the addition of colchicine to the solution. The end was allowed to reach a steady state of assembly in the absence of TuDrug, after which the mol fraction of TuCol in the solution was changed (through the addition of colchicine and assuming immediate binding), keeping the total concentration of dimer (C_t) constant. The conversion of soluble tubulin into TuCol induces a shortening of the microtubule. At low levels of TuCol the length of the microtubule is reduced to a new steady-state value, at which the end switches randomly between states of shortening and inhibited growth (results not shown). At higher mol fractions of TuCol in solution, the new steady state is not established within 1 h after the addition of TuDrug. After an initial high rate of shortening, the rate slows. At very high levels of TuCol in solution, partial or complete disassembly is observed, depending on the total concentration of tubulin in the system. Clearly there is good qualitative agreement between the calculations and the data of Figure 1(a). In particular, the observed initial phase of fast disassembly followed by a longer period of slow disassembly is reproduced well. Figure 5(d) shows the effect of the addition of the drug on C_s. Again, the result is in good agreement with the observations in Figure 1(b). Different cases are distinguished: in the simple model, the amount of drug-induced disassembly is related to the strength of the inhibition (value of f) in a straightforward manner; in the more elaborate model, a relatively small amount of disassembly is induced despite the strong inhibition of microtubule growth.

The calculated $J_{on}(C)$ diagram for GTP/GDP-microtubules is shown in Figure 5(e). The insert shows the effect of TuCol on the critical concentration, as determined by $J_{on}(C) = 0\ \mu m/min$. According to the $J_{on}(C)$ diagram, the presence of TuCol induces a considerable increase in the critical concentration (=

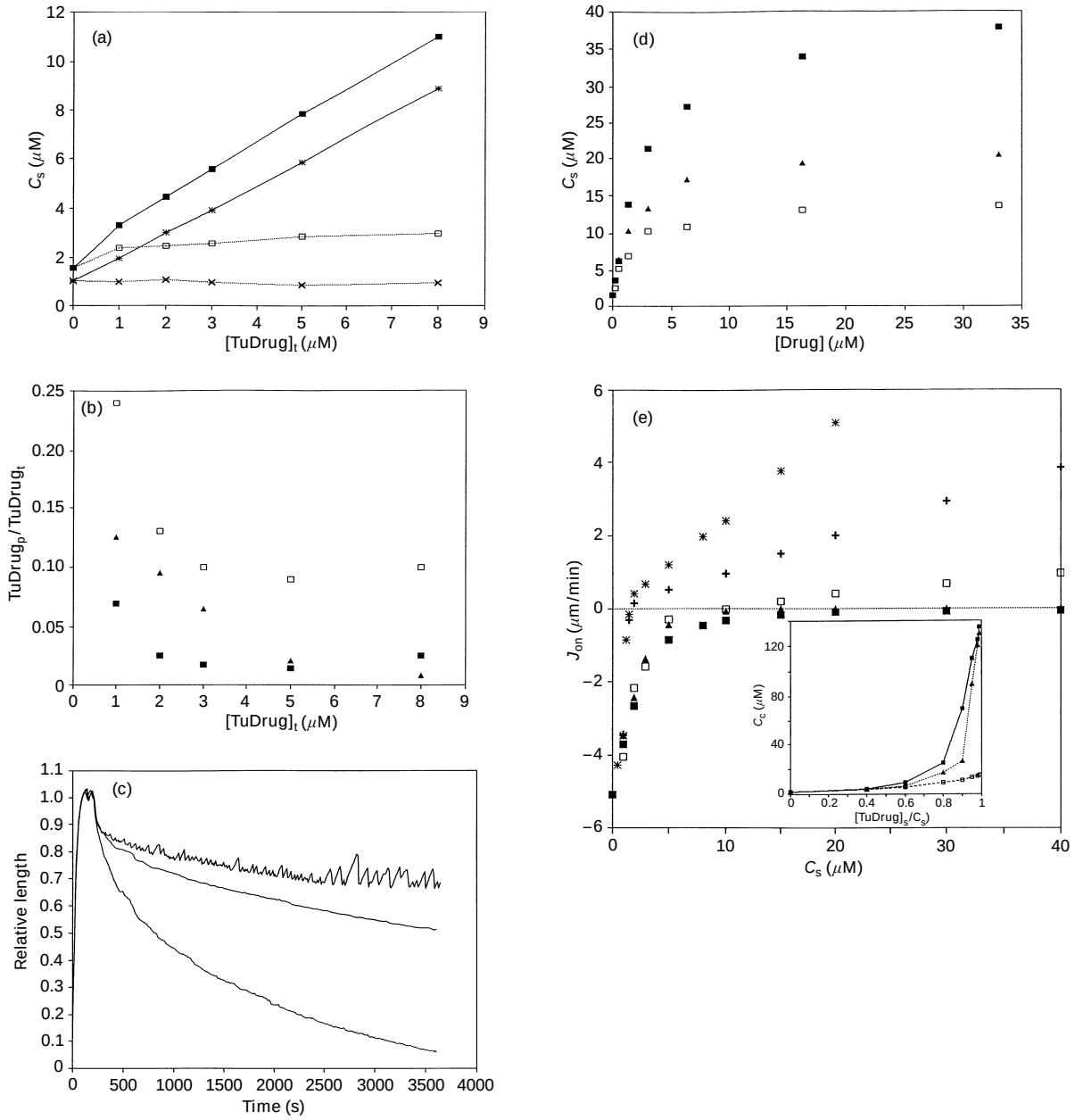

Figure 5 Simulation of the dynamic response of microtubules on addition of TuCol

The method of Martin et al. [31] was used to simulate the dynamic response of the β-out end of a 13-protofilament A-lattice to the addition of TuDrug. (**a**) Effect of the addition of a specified amount of TuDrug ([TuDrug]$_t$) to the reaction mixture on C_s (= [Tub]$_s$ + [TuDrug]$_s$; solid lines) and unliganded dimer in solution, [Tub]$_s$ (dotted lines), for steady-state microtubules (■, □) and equilibrium microtubules (*, ×) ($f = 100$). (**b**) Efficiency of incorporation of TuDrug into the lattice [expressed as the TuDrug in the polymer phase (TuDrug$_p$) incorporated 1 h after addition divided by the total TuDrug added (TuDrug$_t$)]: □, $f = 10$; ■, $f = 100$; ▲, $f_1 = 10/f_2 = 100$. (**c**) Dynamic length change of a single microtubule at steady state upon saturation of 99% of soluble dimer with drug: top curve, $f = 10$; bottom curve, $f = 100$; middle curve, $f_1 = 10/f_2 = 100$. (The length is normalized against the average length at steady state, which is 4.7 μm.) (**d**) Effect of saturation of soluble dimer with drug: C_s as a function of colchicine concentration 1 h after addition of the drug: □, $f = 10$; ■, $f = 100$; ▲, $f_1 = 10/f_2 = 100$. (**e**) Effect of TuDrug on the net growth rate over a long time, J_{on}, of GTP/GDP-microtubules as a function the total concentration of tubulin in solution, C_s. The $J_{on}(C)$ curves were calculated in the absence of TuDrug (*), and in the presence of 0.5% TuDrug, $f = 100$ (+); 99% TuDrug, $f = 10$ (□); 99% TuDrug, $f_1 = 10/f_2 = 100$ (▲); and 99% TuDrug, $f = 100$ (■). Insert: effect of TuDrug on the critical concentration, C_c [with $J_{on}(C_c) = 0$]: □, $f = 10$; ■, $f = 100$; ▲, $f_1 = 10/f_2 = 100$.

$[Tu]_s + [TuCol]_s$ at steady state, where $[TuCol]_s$ is the concentration of TuCol in solution), and causes strong suppression of microtubule growth. In this respect, no qualitative difference exists between the responses of GTP/GDP- and p[NH]ppG-microtubules (results not shown). The $J_{on}(C)$ diagram also indicates that there exists a wide range of C_s values below the critical concentration where disassembly is extremely slow. This is consistent with the continuous slow disassembly observed in Figure 1(a), and results from the high number of addition reactions of both tubulin and TuDrug molecules to the end at high concentrations of dimer in solution. Thus, although the critical concentration has been increased considerably by the addition of TuDrug, for kinetic reasons disassembly is extremely slow.

DISCUSSION

In the present work, we have examined the response of steady-state and equilibrium microtubules to the addition of TuDrug and free drug. Previously, the effects of TuCol on microtubule dynamics were reproduced [14] by assuming that: (1) the affinity of a dimer for a microtubule end depends on the E-site nucleotide content of adjacent subunits; (2) the addition of the dimer triggers hydrolysis of the E-site GTP in the previously terminal subunit; (3) TuCol adds to the lattice with the same affinity as drug-free dimers; and (4) the affinity for sites adjacent to a terminal TuCol is reduced in one helical lattice direction. The observations reported here are reproduced by incorporating these principles into the established model [31].

Significance of colchicine lattice sites in microtubule dynamics

The affinity of colchicine for microtubule lattice wall sites ($K_{MT} \geqslant 300\ M^{-1}$; Figure 3) is 10^4 times lower than its affinity for free tubulin [32–36]. Given this large difference in affinity, a linked function relationship predicts the complete disassembly of microtubules in the presence of excess drug (if TuCol does not bind to the microtubule ends). The fact that this does not happen within the timescale of the observations emphasizes the importance of the interactions of TuCol with the microtubule end. This conclusion is supported by experimental data (Figure 1b) showing that the apparent plateau in the concentration of soluble dimer (C_s) is established at $\sim 20\ \mu M$ colchicine, consistent with the known affinity of TuCol for a microtubule end (K_d 0.38 μM [9] or 0.16 μM [11]). Thus the response of the microtubules to addition of the drug is determined by the effects of TuCol on the microtubule ends, and the direct interaction of colchicine with the microtubule wall does not contribute significantly to these effects.

The affinity of colchicine for lattice sites is similar to that of tropolone and trimethoxyphenol (the constituent rings of colchicine) for tubulin dimers [34,37], suggesting that a part of the colchicine binding site on tubulin remains available when the dimer is incorporated into the microtubule lattice.

Effects of TuCol on equilibrium and steady-state microtubules

The effects of TuCol can, in principle, be considered in terms of the co-assembly of TuCol and unliganded tubulin into microtubules. The ability of two species to co-polymerize is expressed thermodynamically as a decrease in the critical concentration of both [38]. Since pure TuCol microtubules have a very much higher critical concentration than pure tubulin microtubules, the addition of TuCol to pure tubulin microtubules at steady state should lead to a slight decrease in the free tubulin concentration, $[Tu]_s$. In fact, this addition caused a significant increase in $[Tu]_s$ (Figure 1c). This is because tubulin-GDP-containing microtubules are only stabilized by the continuous addition of tubulin-GTP to their ends. The TuCol-induced inhibition of addition reactions interferes with this process and increases the likelihood that microtubules will disassemble, hence the increase in $[Tu]_s$. The microtubules are restabilized by addition reactions (including those involving TuCol) at a new, and higher, dimer concentration, C_s ($= [Tu]_s + [TuCol]_s$) (see below). When added to p[NH]ppG-microtubules (Figure 1c), TuCol simply accumulates in the soluble phase.

Incorporation of TuCol into steady-state microtubules

In agreement with other reports [12,17,18], the level of TuCol incorporation (Figure 2) was two orders of magnitude higher than the typical microtubule number concentration (nM), i.e. more than can be accounted for by complete blocking of the ends with TuCol. The co-assembly of tubulin and TuCol in a microtubule requires two processes: (1) the addition of TuCol to the end, and (2) its subsequent incorporation into the lattice. Although TuCol adds readily to microtubule ends [9,11], the evidence of Figures 1(c) and 2 suggests that its subsequent incorporation into the lattice is unfavourable. According to the proposed model (Figure 4), this is because step 2 requires the addition of a dimer to an inhibited site. Although this step is energetically unfavourable, it does occur with finite likelihood. The incorporation of TuDrug is more likely if the frequency of bimolecular additions (including those involving TuCol) is high, i.e. at high dimer concentrations. However, for any particular concentration C_s, the incorporation of an individual TuCol is more likely when the ratio of TuCol to unliganded tubulin in solution is low; this is because adding TuCol simply creates new inhibited sites. The data of Figure 2 support these views. These arguments also explain the lower level of incorporation of TuCol observed here, where C_s is close to the critical concentration C_c, compared with previous reports in which microtubule fragments were elongated to steady state in the presence of TuCol (i.e. $C_s \gg C_c$ [12,18]). This observation is readily reproduced by simulations (Figure 5b), which also indicate (as expected) that high levels of TuDrug are incorporated when the subsequent inhibition of addition reactions is weak.

Drug-induced disassembly and substoichiometric inhibition

The addition of excess drug to preassembled microtubules induces only partial disassembly (Figure 1a and [5]). When the solution is cooled to 4 °C, normal disassembly is observed, but no reassembly of the microtubules occurs upon reheating to 37 °C. Drug-induced microtubule disassembly is reproduced in Figure 5(c), and the calculated $J_{on}(C)$ diagram (Figure 5e) offers a rationalization of these phenomena. The critical concentration ($= [Tu]_s + [TuCol]_s$), where no net growth or disassembly is observed, is increased significantly when TuCol is present. The addition of colchicine to steady-state microtubules produces TuCol, and disassembly is induced. The free dimer concentration increases, so the frequency of bimolecular addition reactions is therefore increased, and the rate of disassembly is reduced. The $J_{on}(C)$ diagram suggests that disassembly may become very slow, and, in practice, a new steady state may not be established within the observation time. Whether or not complete disassembly will be observed in the presence of excess drug depends on the total tubulin concentration in the system, C_t, and the value of the disassembly rate constant, k_{diss}, given by the intercept of $J_{on}(C)$ with the ordinate. In the presence of reagents which decrease disassembly rates (e.g. glycerol and microtubule-associated proteins), or in the case of p[NH]ppG-microtubules which have a very low k_{diss}, the extent of disassembly will be reduced.

The $J_{on}(C)$ diagram also indicates that TuCol is an efficient inhibitor of microtubule growth. For a 100-fold reduction in the site-specific association rate constant ($k_{+,XY}$) for addition to the inhibited site, the overall growth rate constant (k_{ass}) of the end is reduced by a factor of 3 by the presence of only 0.5 % TuCol in solution, and by a factor of 20 by 10 % TuCol (cf. Sternlicht and Ringel [12]). Low levels of TuCol relative to total tubulin are known to inhibit microtubule assembly completely [3,4], a phenomenon known as substoichiometric inhibition [8,23]. Substoichiometric levels of the drug may, however, represent high concentrations of TuDrug relative to the microtubule number concentration (nM). Given the affinity of TuCol for microtubule ends, they will be $\sim 80\ \%$ saturated by 1 μM TuCol. Both

experimental observations [12–15,39] and the calculated $J_{on}(C)$ diagram indicate that this leads to the effective inhibition of growth. The substoichiometric effect occurs because of the difficulty of incorporating TuCol into the microtubule. Simulations indicate that this effect is likely to be similar for GTP/GDP- and p[NH]ppG-microtubules.

Critique of different models: co-operative inhibition

Colchicine binding is believed to induce a conformational change in tubulin [32–34], and tubulin/TuCol co-polymers are known to be non-tubular [19–22]. The addition of TuCol to a microtubule end may therefore produce a lattice defect which is further amplified by TuCol in adjacent positions (i.e. a co-operative effect). Moreover, the growth of individual microtubule ends is effectively inhibited by moderately low concentrations of TuCol [13–15], suggesting a marked decrease in the association rate constant, $k_{+,XY}$, for lattice sites adjacent to TuDrug. On the other hand, the extent of colchicine-induced disassembly is relatively small ([14]; the present work), and steady state (equilibrium) is not re-established within 1 h after addition of the drug (Figure 1), suggesting a strong retardation of microtubule disassembly. Taken separately, each of these properties is readily reproduced in the simplest model. However, the combination of a marked kinetic retardation of disassembly and a substantial inhibition of growth can only be reproduced if the possibility of co-operative effects in the inhibition is incorporated into the model (Figure 5c). The principle of co-operative effects in the inhibition is illustrated in Figure 4, which shows the simplest possible case involving only two adjacent TuDrug molecules. In reality, co-operative effects may involve more TuDrug molecules, and, at high $[TuCol]_s/[Tu]_s$ ratios, may even lead to a complete prevention of co-polymerization. This would resolve the earlier controversy between a TuCol-cap model [39] and a co-polymerization model [12,17].

Recently, Panda et al. [15] suggested that microtubule ends are 'kinetically stabilized' by very low $[TuCol]_s$ values. This suggestion implies that the site-specific dissociation rate constant for a TuCol-containing site in the microtubule lattice is (significantly) reduced. Our observations offer no indication of this site-specific effect. The difficulty with the suggestion of Panda et al. is in rationalizing how such an effect can vanish at higher TuCol concentrations, as clearly shown by the significant increase in $[Tu]_s$ (see Fig. 1b of [14] for the effect in the absence of glycerol). Stabilization of the microtubule lattice by low concentrations of TuCol only seems possible when (1) the affinity of TuCol for the microtubule lattice is higher than the affinity of tubulin-GTP (cf. [9,11]), and (2) the inhibition caused by a single TuCol in the end is weak, and (3) (strong) co-operative effects exist in the inhibition.

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