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Tubulin-Colchicine Complex (TC) Inhibits Microtubule Depolymerization by a Capping Reaction Exerted Preferentially at the Minus End

Lawrence G. Bergen and Gary G. Borisy

Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

The effects of colchicine and tubulin-colchicine complex (TC) on microtubule depolymerization were studied using the axoneme-subunit system described previously [Bergen LG, Borisy GG; J Cell Biol 84:141–150, 1980]. This system allows the independent analysis of the polymerization kinetics at both the plus and minus ends of a microtubule. Depolymerization was induced by isothermal dilution with 10 volumes of an experimental solution containing colchicine, TC, or buffer alone. Colchicine alone (5–100 μM) blocked depolymerization at the minus end, whereas depolymerization at the plus end occurred at almost control rates. A similar effect was produced by TC (0.4:1–1:1 molar ratio to free tubulin). High molar ratios of TC to tubulin (10:1) blocked depolymerization at both plus and minus ends, and intermediate molar ratios of TC:T allowed depolymerization of the plus ends but at attenuated rates. The blockage was not readily reversible; TC-affected ends neither shortened upon dilution nor grew longer upon incubation with additional tubulin. We conclude that TC at suprastoichiometric ratios to tubulin inhibits microtubule depolymerization by a capping reaction and that this effect is exerted preferentially at the minus end.

Key words: tubulin-colchicine, microtubules, depolymerization, antimicrotubule drugs

Colchicine has been used routinely to depolymerize microtubules in vivo [see 1]. In vitro colchicine has been found to complex with tubulin and to inhibit the polymerization of microtubules at levels substoichiometric to the concentration of tubulin protomer [2–7]. Although generally considered to be a tool to remove microtubules, several workers [5,8–11] have reported that suprastoichiometric levels do not induce complete depolymerization of microtubules. Rather, limited or no depolymerization occurred. Resistance to disassembly was one of the observations that led Margolis

Lawrence G. Bergen's present address is Department of Bacteriology, University of Wisconsin, Madison, WI 53706.

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and Wilson [5] to propose that substoichiometric poisoning of microtubule assembly was the result of a TC-induced capping of the assembly end of the microtubule. Although this model has been shown to be untenable as an explanation for the substoichiometric inhibition of assembly [2,4,6,12], we thought the possibility of capping was worth reexploring as an explanation for the suprastoichiometric resistance to disassembly.

The axoneme-subunit system [13,14] allows the positive structural identification of microtubule ends. By measuring time-dependent changes in microtubule length, we are able to separately analyze the end-mediated reactions that occur at each end. In a previous paper, using the axoneme-subunit system [2], we reported that substoichiometric amounts of TC inhibited elongation of plus- and minus-end microtubules equally without affecting dissociation. In this paper, we examine the effects of colchicine and TC at suprastoichiometric levels on microtubule depolymerization. We use the axoneme-subunit system to analyze the action of TC independently at the two ends of a microtubule and show that TC does inhibit microtubule depolymerization by a capping reaction. However, contrary to models suggested previously [5,15] that stipulated the cap to occur solely at the assembly or plus end, we find the inhibitory effect to be exerted at both ends and preferentially at the minus end.

MATERIALS AND METHODS

The preparation of materials and the experimental procedures have been described in detail previously [2,13,14]. Briefly, porcine brain microtubule protein was purified by cycles of assembly and disassembly and was then centrifuged at high speed to attenuate self-nucleation. The resultant microtubule subunits contain a full complement of microtubule-associated proteins (MAPs) as detected by high-resolution gel electrophoresis [Peloquin and Borisy, unpublished data], and the mechanism by which self-nucleation is inhibited remains unknown. Microtubules were polymerized upon axonemal templates isolated from the flagella of Chlamydomonas reinhardtii by incubation at polymerization temperature (30°C). The axoneme-microtubule complexes were then used as the starting point for the depolymerization experiments. Tubulin-colchicine complex (TC) was formed by incubating MAP-free tubulin with fivefold molar excess colchicine at 37°C for 45 min and was then separated from unbound colchicine by molecular sieve chromatography (Sephadex G25 [2]). All dilutions and preparation of solutions were made with PEMG buffer [0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, pH 6.94]. Microtubule polymerization and depolymerization were assayed as changes in length by quantitative electron microscopy. Plus and minus ends were distinguished by morphological criteria. Microtubule depolymerization was induced by isothermal dilution, and the effects of colchicine and TC on depolymerization were studied by addition of these agents at the time of dilution.

RESULTS

To study the action of colchicine on microtubule depolymerization, we first had to polymerize microtubules onto axonemal seeds. This was done by incubating Chlamydomonas axonemes (1–2 × 10^7/ml final concentration) with porcine brain

microtubule subunits (8–11 μM final concentration) at 30°C for 5 min. The template seeded microtubules are polymerized at a linear rate (approximately 2.0 and 0.6 μm/min for the plus and minus ends, respectively and attain respective lengths of approximately 10 and 3 μm by 5 min. After this incubation, depolymerization was induced by isothermal dilution with 10 volumes of an experimental solution. Under these conditions, the microtubule subunit concentration was reduced to below the critical concentration, and net loss of subunits occurred at both microtubule ends. When the dilution was made with PEMG buffer, depolymerization rates at 30°C for the plus and minus ends were 1.0 $\pm$ 0.1 and 0.35 $\pm$ 0.06 μm/min, respectively. These rates were constant with time, and the microtubules shortened to zero length [13]. Because depolymerization by isothermal dilution proceeded at a constant rate and went to completion, analysis of the action of agents on the depolymerization mechanism could be simplified. To study the action of colchicine and tubulin-colchicine complex (TC) we simply added these agents at known concentrations at the time of isothermal dilution and compared the rates of depolymerization to the basal values obtained with buffer alone.

Figure 1 shows the results obtained when the experimental solution contained 100 μM colchicine. The data are plotted as change in length from the average predilution values. As can be seen, depolymerization at the minus end was inhibited totally in the presence of cochicine, whereas depolymerization at the plus end occurred at the same rate as in buffer alone. The same result was obtained at concentrations of colchicine down to 5 μM. Below 5 μM, depolymerization at the minus end was also observed, approaching control rates at 0.1 μM (data not shown).

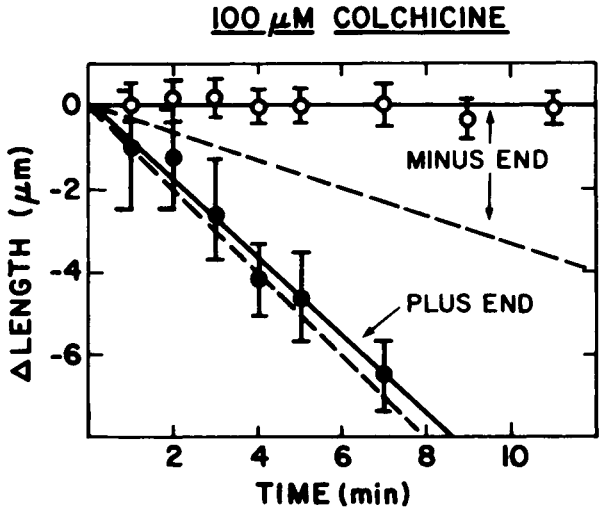

Fig. 1. Inhibition of microtubule depolymerization by colchicine. Microtubules were first polymerized onto axonemal seeds with 8 μM subunits, and the preparation was then diluted with 10 volumes of PEMG containing 100 μM colchicine (solid lines) at 30°C. Length changes for plus (●) and minus (○) end microtubules are plotted versus time. The dashed lines represent the corresponding length changes in the absence of colchicine. Each point corresponds to the mean length of at least 20 microtubules; the error bars represent the standard deviations of length.

The effects of colchicine on depolymerization were remarkable in several respects. First, the drug did inhibit depolymerization, whereas it is customarily used in vivo to promote net depolymerization, presumably by inhibiting the association reaction. Second, the effect was a polar one; one end was inhibited and the other was not. Third, the inhibited end was the minus end, whereas the model proposed for the action of colchicine [5,15] would have it act at the plus end.

To document the stability of the minus-ended microtubules in the presence of colchicine, we show an electron micrograph of the axoneme-microtubule complex in Figure 2. The plus and minus ends of the axoneme are readily identified by the frayed character of the plus end and the intact minus end [13,14]. Prior to the dilution with colchicine, the number (N) and length (L) of microtubules were $N = 12 \pm 4$, $L = 8.5 \pm 1.9$ μm at the plus end, and $N = 9 \pm 2$, $L = 3.0 \pm 0.7$ μm at the minus end. As can be seen in Figure 2, the minus-ended microtubules have the predilution number and length, whereas the plus-ended microtubules depolymerized almost completely; only two short stubs remain.

The inhibitory effects of colchicine on microtubule polymerization have been shown to be mediated by TC [2,4–6]. However, the inhibitory effects of colchicine on depolymerization have not been similarly studied. In the experiments in which axoneme-microtubule complexes were diluted 10× into colchicine, the microtubule subunit concentration was reduced to below the critical value for assembly. However, this was still a sufficient concentration to permit the formation of TC and allow inhibition of depolymerization by TC rather than by colchicine directly.

To test directly whether TC could inhibit microtubule depolymerization, pure tubulin was prepared and complexed with colchicine as described previously [2].

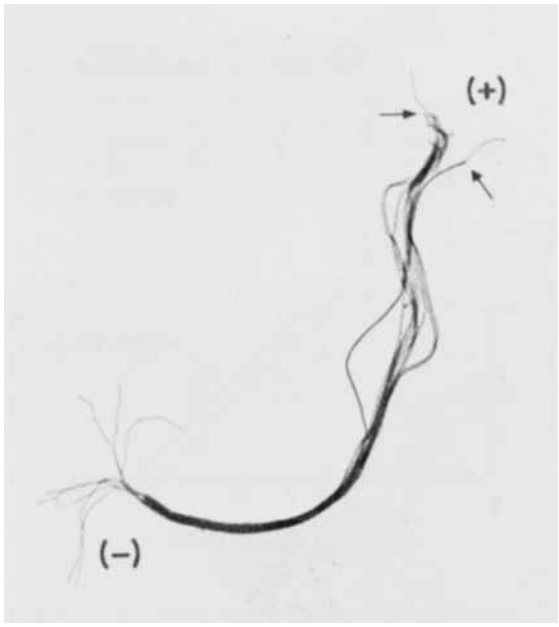

Fig. 2. Electron micrograph of axoneme from the t = 11 min sample of Figure 1. The plus end of the axoneme is identified by the characteristic fraying of the axonemal doublet tubules; the minus end remains intact. Eleven minutes after dilution into 100 μM colchicine at 30°C, almost all the plus-ended microtubules had depolymerized to the axonemes. Two short stubs remain on this axoneme and are indicated by arrows. The minus-ended microtubules have remained unchanged in length. ×5,700.

Because the TC complex has a half-life of 36 hr [16], there was no appreciable colchicine produced during the course of the experiment. Axoneme-microtubule complexes were prepared, isothermally diluted with 10 volumes of PEMG solution containing TC, and analyzed for length changes by electron microscopy. The results of an experiment at 1.2 μM TC final concentration (Fig. 3) were similar to those obtained with 5–100 μM colchicine. The plus-ended microtubules depolymerized, whereas the minus-ended microtubules remained unchanged in length over the course of the experiment. Thus TC preferentially inhibited depolymerization at the minus end. As with dilution into colchicine, the number of minus-ended microtubules also remained constant after dilution, indicating that these tubules were homogeneous in their sensitivity to TC (data not shown). However, the plus end was affected; the rate of depolymerization was only 75% that of the control rate.

To determine whether this reduction from control rate was significant, we tested the dependence of depolymerization inhibition on the TC:T (tubulin) ratio (Fig. 4). In that the tubulin concentration, after dilution, was approximately 1 μM, an experiment at 1 μM TC means a dilution to a TC:tubulin molar ratio of ~ 1. As is shown in Figure 4, at a TC:T ratio of 0.2, the plus end was not affected detectably, whereas the minus end was inhibited 50%. Also, at a TC:T rate of 1 depolymerization at the minus end is totally inhibited, whereas the plus end is inhibited only 60%. Therefore, although TC at sufficiently high concentrations blocks depolymerization of both the plus and minus end, it acts preferentially on the minus end.

We also examined the reversibility of the inhibitory action of TC (Fig. 5). Microtubules were first polymerized onto axonemes, and then TC was added. The reversibility of the inhibition by TC was then tested by isothermal dilution of the preparation into buffer alone, buffer containing microtubule subunits, or buffer containing additional TC. If the action of TC on the microtubules was readily reversible, the microtubules diluted into buffer alone would shorten, the ones mixed with subunits would grow longer, and the ones mixed with TC would remain the same length. The results show that the minus-ended microtubules remained the same length in all three cases. Similar results were obtained at inhibiting concentrations of

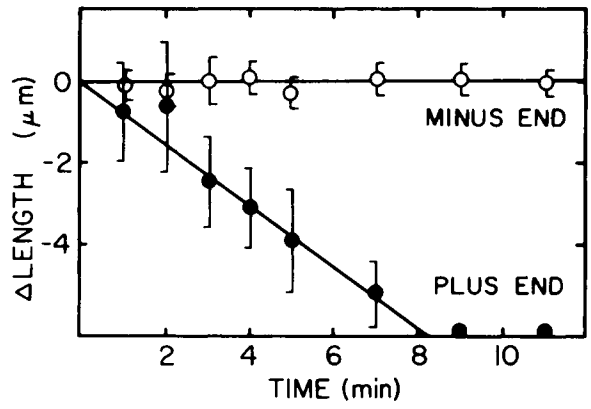

Fig. 3. Inhibition of microtubule depolymerization by tubulin-colchicine complex (TC). The protocol for the experiment was the same as that described for Figure 1, except TC was used instead of colchicine. The changes of length of plus (●) and minus (○) end microtubules are plotted against time after dilution into PEMG containing 1.2 μM TC at 30°C.

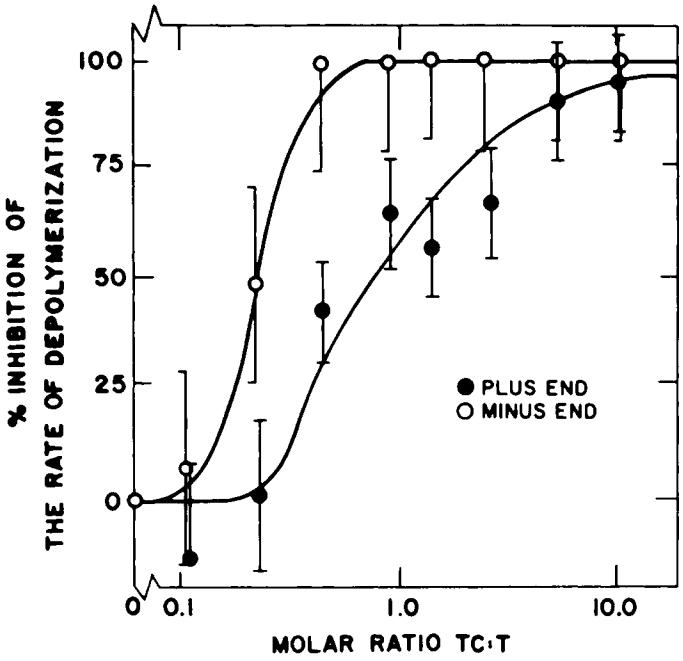

Fig. 4. Concentration dependence of inhibition of depolymerization by TC. Axoneme-microtubule complexes were diluted into PEMG with various concentrations of TC at 30°C. Percent inhibition of depolymerization was calculated as 100 × (1 − observed rate/control rate). Percent inhibition is plotted for both plus (●) and minus (○) ends versus the log of TC concentration. Concentration of free tubulin dimers (T) in all cases was approximately 1 μM.

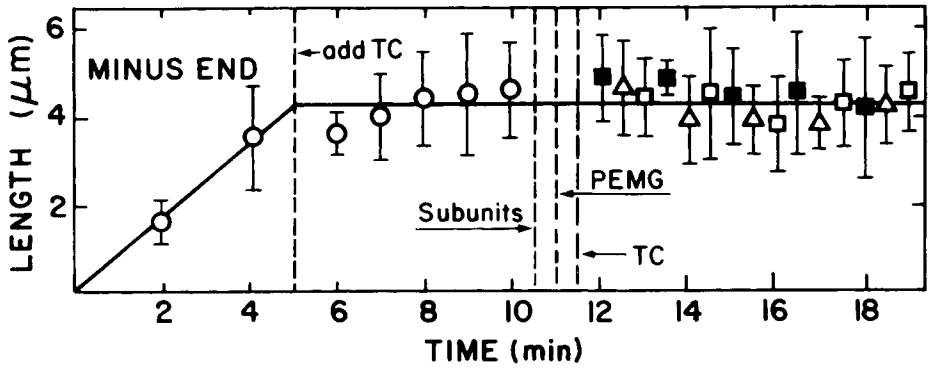

Fig. 5. Stabilization of minus-ended microtubules by TC and resistance to reversal. At time 0, axonemes were mixed with 8 μM subunits at 30°C, and polymerization was allowed to occur for 5 min; then TC (3.5 μM final concentration) was added to stabilize the minus end (○). The length of minus-ended microtubules was followed further after 11-fold dilutions into either 12 μM T (■, t = 10.5 min), PEMG (△, t = 11 min), or 5 μM TC (□, t = 11.5 min). Dashed lines represent the various times of dilution and addition of TC.

TC for the plus-ended microtubules (data not shown). The results suggest that under these conditions TC forms a cap that blocks both addition and loss of subunits.

DISCUSSION

The major findings of this paper are as follows: 1) The resistance of microtubules to depolymerization by suprastoichiometric levels of colchicine is mediated by TC. 2) The stabilizing effects of TC are exerted at both ends of a microtubule but preferentially at the minus end. 3) Suprastoichiometric ratios of TC:T cap the ends of microtubules in the sense that the ends are unable either to gain or to lose subunits and that this loss of reactivity is not readily reversible.

The inhibition of microtubule depolymerization by suprastoichiometric amounts of colchicine is similar to the inhibition of microtubule elongation by substoichiometric amounts of colchicine; for both cases, colchicine first binds to free tubulin, and it is the TC that appears to mediate the effects on microtubule stability. The TC complex is very stable, with a half-life of 36 hr [16]. Thus, during the course of the experiments, the effects seen in solutions containing purified TC are not attributable to free colchicine. Furthermore, TC is more potent than colchicine. TC at 1.2 μM caps the minus end and at 10μM (TC:T molar ratio of 10.0, see Fig. 4) caps both minus and plus ends, whereas colchicine at 1.2 μM caps neither end and at 10 μM caps only the minus end. However, our results do not exclude a direct effect of colchicine on microtubules. We can conclude that a direct binding to microtubules is not necessary for stabilization, and, if it does occur at all, it would be occurring competitively with demonstrated (sufficient) binding of TC.

Whether TC inhibits the dissociation of GTP-bound tubulin, GDP-bound tubulin, or both is unknown. Similarly, it is not known if the two microtubule ends differ in GTP content during depolymerization. However, dynamic instability (the rapid total depolymerization of select microtubules) reported to be caused by the loss of a "GTP-cap" [17]was not detected in any of our experiments. Mitchison and Kirschner [17] have suggested that MAPs would dampen this phenomonon, which probably explains stability in our system. An indication that MAPs are involved in the stabilizing effects of TC is the report [18] that colchicine added to microtubules formed from pure tubulin induced rapid and complete depolymerization in contrast to the results of others (cited in the Introduction) with MAP-microtubules in which only limited depolymerization was reported.

Although TC can stabilize both ends of a microtubule, it does so in a polar manner. This is in contrast to the substoichiometric poisoning of assembly, which is nonpolar [2]. One possible explanation is that the polarity of stabilization reflects the inherent structural polarity of the tubulin subunits in the microtubule [19]. Another possibility is that the polarity of stabilization results from a kinetic polarity (i.e., in that depolymerization from the minus end is slower than from the plus end, there is an increased probability for cumulative effects to take place at the minus end) that arises from the interaction of tubulin and microtubule-associated proteins.

We have shown that suprastoichiometric poisoning is not readily reversible. One possibility for this lack of reversibility is a cooperative mechanism involving multiple binding sites (for either T or TC) at the end of a microtubule. Increased ratios of TC:T would favor the occupancy of adjacent sites by TC. Stabilization

would be achieved if the dissociation rate constant for TC was greatly reduced when a neighboring site was also occupied by TC.

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