How Taxol Modulates Microtubule Disassembly*

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Measurement of the affinity of microtubules for the anti-cancer drug taxol is problematic, because microtubules are not stable at the very low concentrations required to detect taxol dissociation. We have circumvented this problem by using the GTP analogue GMPCPP (guanylyl α,β-methylene-diphosphonate), which renders microtubules sufficiently stable to allow binding studies with natsaturating concentrations of taxol. A Kd value equal to about 10 ns was estimated from the effect of taxol concentration on the dilution-induced disassembly rate and on the binding of [3H]taxol. With GTP-microtubules the Kd value for taxol binding by tubulin-GDP subunits in the core of the microtubule appears to be comparable with that of GMPCPP-microtubules. However, the stabilizing effect of the drug bound to tubulin subunits that arrive at ends of disassembling microtubules is attenuated by a two-step reaction sequence in which taxol dissociates (k = 30 s⁻¹), followed by a rapid (k = 1000 s⁻¹) loss of the taxol-free tubulin subunit. This sequential reaction can be disrupted by high (micromolar) concentrations of taxol, which react rapidly with tubulin subunits at the ends of microtubules (k = 2 x 10⁶ M⁻¹ s⁻¹). The inhibitory effect of taxol on microtubule disassembly at concentrations a thousand-fold greater than the Kd value suggests the desirability of using high taxol concentrations in chemotherapy with this compound.

Microtubules bind the drug taxol, a natural product from the yew (Taxus brevifolia) (1). Use of taxol for cancer therapy, as well as efforts to develop improved taxol derivatives, would benefit from a detailed understanding of the kinetics and equilibrium for taxol binding. Earlier, microtubules were found to bind 100% of added taxol at taxol concentrations between 0.2 and 1.2 μM (2). These results indicate a Kd value of taxol substantially less than 0.2 μM, by some factor that remains to be determined. Binding experiments at lower taxol and microtubule concentrations are rendered problematic by the fact that microtubules are not stable when diluted to 0.2 μM tubulin in microtubules.

In contrast, microtubules assembled with GMPCPP are extremely stable, and have a significant lifetime even after extreme dilution (3). In the present study, GMPCPP-microtubules were found to be sufficiently stable at ns concentrations to allow equilibrium for taxol binding to tubulin subunits, including those at microtubule ends. The effect of taxol concentration on the dilution-induced disassembly rate and on the extent of binding of [3H]taxol allowed us to estimate a Kd value of about 10 ns. Taxol binding to the tubulin-GDP subunits that form the core of microtubules assembled with GTP is apparently comparable to that with GMPCPP-microtubules. However, taxol is not at equilibrium with terminal tubulin-GDP subunits, because taxol-free subunits dissociate from ends faster than taxol (at concentrations near Kd) is bound by these subunits. The escape of taxol-free tubulin subunits from ends of microtubules whose tubulin-GDP core is saturated with taxol can be suppressed by using taxol concentrations that are 1000 times greater than Kd. From these results, we infer that in chemotherapy a maximal effect is to be expected when the taxol concentration is sufficient both to saturate the core of the microtubule and to provide enough free taxol to capture taxol-free subunits efficiently at microtubule ends. The observed inhibition of disassembly by very high taxol concentrations can account for the observation that intracellular taxol concentrations that exceed the tubulin concentration are required to increase the amount of tubulin in polymer in HeLa cells (4). The assay described here for study of taxol binding with GMCP CPP-microtubules may prove useful in developing improved taxol derivatives.

EXPERIMENTAL PROCEDURES

Materials—Beef brain tubulin, GMPCPP, and [γ-32P]GMPCPP were prepared as described (3, 5). [3H]Taxol was obtained from Morevic Biochemical; 99% of the tritium was shown to be in taxol by analysis based on the Gibbs phase rule (6). This is a highly sensitive method and the most accurate method for determining taxol purity.

Methods—All reactions were at 37 °C in BRB80 buffer (80 mM NaCl, 0.5 mM MgCl2, 1 mM EGTA, pH 6.8); reaction mixtures with microtubules were made up and handled in a room at 37 °C. GMPCPP-microtubules were prepared by two cycles of thermal induced assembly and disassembly with 10 μM tubulin, using 1 μM GMPCPP in the first cycle and 100 μM [32P]GMPCPP in the second cycle. Microtubules were isolated using a Beckman Airfuge (3-10 min, 30 p.s.i.).

In studies of the effect of taxol on the rate of dilution-induced disassembly of GTP-microtubules, these were formed by a 30-min incubation of 50 μM tubulin, 44 mM acetyl phosphate, 0.36 unit/ml acetyl kinase, and a trace amount of [γ-32P]GTP. A 20:1 aliquot of this mixture that had been diluted with 1 ml of 5 μM taxol was layered on a 4-mL cushion of 40% glycerol in BRB80 buffer. After microtubules were isolated by centrifugation (50,000 rpm, T50 rotor, 37 °C), the pellet was resuspended in 2 ml of buffer containing alkaline phosphatase (1 unit/ml) and taxol, which was transferred to a siliconized glass tube.

The kinetics for dilution-induced disassembly were measured from the rate of release of [32P]P1, formed in Reactions 1 and 2.

\[
[32P]GMPCPP-microtubule \rightarrow \text{[32P]GMPCPP-tubulin subunit}
\]

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Taxol Reaction with Microtubules

**RESULTS AND DISCUSSION**

**Reaction of Taxol with GMPCP-Microtubules**—Interaction of taxol with GMPCP-microtubules was detected by its effect on the rate of disassembly after a 10,000-fold dilution (Fig. 1).

The resultant concentration of taxol in microtubin was only 0.73 nM, so that saturation was possible with low concentrations of taxol. The observed disassembly rate could be accounted for by assuming that the dissociation rate is 0.65 s⁻¹ for taxol-free tubulin subunits and 0.03 s⁻¹ for taxol-tubulin subunits, with taxol binding to a site on the 5 nM  

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| Experiment | GMP | Taxol | Tubulin | Pellet | Stoichiometry |
|------------|-----|------|--------|-------|--------------|
| A³ | GTP | 10.0 | 5/10 | 2.16 | 3.17 | 0.68/0.78 |
| A³ | GMP | 10.5 | 5/10 | 2.75 | 4.36 | 0.63/0.69 |
| B² | GTP | 10.0 | 5.0 | 3.1 | 3.1 | 1.00 |
| B² | GTP | 1.0 | 0.5 | 0.21 | 0.22 | 0.95 |

³ Nuclease used for microtubul assembly.
³ Reactions were run with both 5 and 10 μm tubulin. [³H]Taxol was incubated with microtubules for 10 min, and the protein and radioactivity were determined in triplicate in the supernatant and pellet after centrifugation in an Airfuge.
³ Microtubules assembled with 1 nM GTP and 50 μm tubulin were made 50 μm in taxol and incubated for 10 min. This mixture was diluted 10-fold into 5 μm taxol and incubated an additional 10 min. Aliquots were then added to [³H]taxol contained in either a 5% volume or a 9-fold greater volume, after 90 s microtubules were isolated with an Airfuge. Radioactivity and protein were measured in the resultant pellet.

The taxol binding isotherm appears to saturate with only about 70% saturation was possible with low concentrations of taxol. In another experiment with 1310 nM tubulin in microtubules, the concentration of taxol was 20 nM, and the protein and radioactivity were measured in the resultant pellet.

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Airfuge rotor.

in our binding reactions; perhaps a fraction of the microtubule regard to the taxol dissociation rate, since tubulin-GMPCPP they dissociate from the microtubule end. The recapture of the end before tubulin subunit dissociation reduces the rate; under these conditions, the rate-limiting step for disassembly is loss of taxol-free subunits. The agreement between the values determined from kinetic and thermodynamic measurements proves that taxol is at equilibrium with all subunits, including terminal subunits; the rate was calculated from the equation: rate = $k_+ \times f_s + k_- \times f_i$. In this case, the curve resembles a simple titration curve with a half-maximal decrease in rate when 50% of the subunits have bound taxol. The data at high taxol concentrations rule out the mechanism described by Curve B.

unable to account for the less than 100% recovery of $[^{3}H]$taxol in our binding reactions; perhaps a fraction of the microtubule pellet is lost because of turbulence during deceleration of the Airfuge rotor.

The agreement between the $K_d$ values determined from kinetic and thermodynamic measurements proves that taxol is at equilibrium with tubulin subunits in the microtubule. For taxol in solution to be at equilibrium with terminal subunits, it is required that both taxol dissociation from and association with terminal subunits be faster than subunit dissociation. With regard to the taxol dissociation rate, since tubulin-GMPCPP subunits dissociate from microtubule ends at a rate of only 0.1–1 s$^{-1}$ (3), maintaining equilibrium requires that taxol dissociate at a rate $>10$ s$^{-1}$. With regard to the taxol association rate, for this rate to exceed the subunit dissociation rate in the nx concentration range, the second-order rate constant for taxol addition must be on the order of $1 \times 10^9$ M$^{-1}$ s$^{-1}$, so that addition of taxol occurs at rates $>10$ s$^{-1}$, even at the lowest taxol concentration. Results described next prove that both the taxol dissociation and association rates with microtubules are sufficiently large to allow the taxol-GMPCPP-microtubule reaction to be at equilibrium.

Reaction of Taxol with GTP-Microtubules—Because microtubules formed with GTP are more dynamic than GMPCPP-microtubules, there is an important difference in the mechanism for taxol inhibition of the disassembly rate. Unlike GMPCPP-microtubules, where taxol-free subunits at the ends of microtubules wait around long enough before dissociating to rebind taxol from solution, taxol-free subunits at ends of GTP-microtubules are likely to dissociate before they rebind taxol. This
rapid dissociation is expected to continue until a taxol-containing subunit is discovered at the microtubule end; as a result, terminal tubulin subunits will be enriched with taxol compared to interior subunits (Fig. 3). Under these conditions, the observed rate constant for disassembly (k_{obs}) is described by the relationship in Equation 1,

\[ \frac{1}{k_{obs}} = f_+ / k_+ + f_- / k_- \]  

(Eq. 1)

where f_+ and f_- are, respectively, the fraction of tubulin subunits in the microtubule without and with bound taxol (f_+ = (K_p/(K_d + taxol)) and f_- = (1 - f_+)); k_+ is the rate of dissociation of taxol-free tubulin-GDP subunits (1000 s^{-1}; Ref. 5) and k_- is the rate of dissociation of terminal subunits that arrive at microtubule ends with bound taxol. Substitution into Equation 1 reveals that if taxol-tubulin subunits dissociation is very slow, there is an enormous potential for taxol to retard disassembly. For example, if taxol reduces the rate of subunit dissociation 100-fold, then the disassembly rate is reduced 2-fold when only 1% of subunits contain taxol.

The disassembly rate with GTP-microtubules was too fast to study with taxol concentrations < 10 nm. The markedly reduced rate observed with 10 nm taxol was little changed by taxol concentrations up to 100 nm (Fig. 4A). The invariance of the rate in this concentration range is consistent with the 5–20 nm K_d determined for taxol binding to GMPCPP-microtubules. It was, therefore, of interest that the disassembly rate decreased 10-fold when the taxol concentration was increased from 500 to 5000 nm (Fig. 4). This decrease apparently did not result from an increase in the amount of bound taxol, since the stoichiometry for taxol binding was constant when taxol was varied in the micromolar concentration range (experiment B in Table I).

The rate decrease at very high taxol concentrations would appear to result because the path for disassembly of taxol-stabilized GTP-microtubules involves rate-limiting dissociation of taxol, followed by rapid loss of the resultant taxol-free subunit. The observed rate for microtubule disassembly when taxol dissociation is rate-limiting5 (i.e. with 10–100 nm taxol) gives the rate constant for taxol dissociation; this is equal to 30 s^{-1}. A k_{obs} of < 1.37 s^{-1} was previously found for disassembly in 50 μm taxol (7). This lower rate probably resulted from the inhibition by taxol concentrations > 0.5 μm, as described in Fig. 4.

5 Inspection of the equation describing k_+ in the legend to Fig. 4B shows that the rate is equal to k_{taxol} when k_+ > k_{taxol}(taxol). A different rate-limiting step holds at taxol concentrations sufficient that k_+ < k_{taxol}(taxol). In this case, k_{taxol} = (K_p(taxol))/k_+ and loss of taxol-free subunits (k_-) is rate-limiting.

It was possible to determine the rate constant for taxol binding to tubulin subunits at microtubule ends from the inhibition of the disassembly rate induced by 0.5–5 μm taxol. Inhibition in this concentration range apparently results because the rate of taxol binding is sufficiently rapid so that taxol-free subunits are captured before they can dissociate; this corresponds to a change in rate-limiting step so that dissociation of taxol-free tubulin subunits becomes rate-limiting. Since 0.5 μm taxol reduced k_{obs} 2-fold, compared to the rate with taxol at 25–100 nm, the rate for taxol binding must be equal to that for subunit dissociation; the rate of taxol binding is therefore 1000 s^{-1}. This rate with 0.5 μm taxol corresponds to a second-order rate constant equal to 2 \times 10^{9} M^{-1} s^{-1}.

The K_p for reaction of taxol with GTP-microtubules can be calculated from the ratio of the constant for taxol binding and dissociation described above; this is equal to 30 s^{-1/2} \times 10^{9} M^{-1} s^{-1} = 15 nm. This value agrees with the 5–20 nm value determined with GMPCPP microtubules (Fig. 1 and 2); however, these results differ dramatically from the 790–870 μm value for K_p reported previously (2). In the earlier study, microtubules were titrated with [3H]taxol under conditions where 100% of the added taxol bound to microtubules; the concentration of taxol sufficient to saturate half the active tubulin concentration was mistakenly taken to be the equilibrium constant. That is, microtubules assembled with microtubule protein containing 3.08 μm tubulin were found to bind 0.53 taxol/tubulin dimer in microtubules at saturating concentrations of taxol, corresponding to binding of 1650 nm taxol. When 815 nm taxol was added the concentration of tubulin subunits with and without bound taxol was equal. However, the concentration of free taxol was near zero, rather than equal to 815 nm, as required if K_p is equal to 815 nm.

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REFERENCES
1. Schaff, P. B., Fant, J., and Horwitz, S. B. (1979) Nature 277, 665–667
2. Parsons, J., and Horwitz, S. B. (1981) J. Cell Biol. 91, 479–487
3. Hyman, A. A., Salié, S., Drechsel, D. N., Unwin, N., and Mitchison, T. J. (1992) Mol. Biol. Cell 3, 1155–1167
4. Jordan, M. A., Torres, R. J., Thresher, D., and Wilson, L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 9552–9556
5. Caplow, M., Kuhlen, R., Shanks, J., Walker, R. A., and Salmon, R. D. (1989) Biochemistry 28, 8136–8141
6. Butler, J. A. V. (1940) J. Gen. Physiol. 24, 189–202
7. Caplow, M., and Zeeberg, B. (1982) Eur. J. Biochem. 127, 319–324

The microtubule protein concentration was 4.4 μm, and this contained 70% tubulin.