Suppression of Microtubule Dynamics by LY290181
A POTENTIAL MECHANISM FOR ITS ANTIPROLIFERATIVE ACTION*

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LY290181, 2-amino-4-(3-pyridyl)-4H-naphtho(1,2-b)pyran-3-carbonitrile, is a potent antiproliferative compound that blocks cells in the G2/M phase of the cell cycle by an apparent action on microtubules. In the present work we found that LY290181 bound to tubulin with high affinity (1 mol of LY290181 per mol of tubulin dimer; \(K_a = 3.8 \times 10^5 \text{ M}^{-1}\)) and that it did not appear to bind at the colchicine or vinblastine-binding sites. LY290181 strongly stabilized microtubule dynamics as determined by video microscopy. It reduced the rate and extent of growing and shortening, reduced the catastrophe frequency and increased the rescue frequency, and increased the percentage of time the microtubules spent in an attenuated state. At the lowest frequency, and increased the percentage of time the microtubules were in shortening states, a behavior termed dynamic instability (11–15). Taxol, which increases microtubule polymerization, similarly inhibited cell proliferation at prometaphase of mitosis (11, 14–19).

In a separate investigation we have found that LY290181 inhibited proliferation of mammalian cells at prometaphase with half-maximal inhibition occurring at \(-20–40 \text{ nM}\) LY290181 and also inhibited microtubule polymerization and depolymerized preformed microtubules in vitro. In the present study, we determined the effects of LY290181 on microtubule polymer mass and dynamics at steady state by video microscopy and characterized the binding site for LY290181 in tubulin. We found that LY290181 binds to tubulin at a novel site distinct from those of vinblastine and colchicine but stabilizes microtubule dynamics in a manner similar to that of these two drugs.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain microtubule protein was isolated without glycerol by three cycles of polymerization and depolymerization. Tubulin was purified from the microtubule protein by phosphocellulose chromatography as described previously (20). The tubulin solution was quickly frozen as drops in liquid nitrogen and stored at \(-70^\circ \text{C}\) until used. Protein concentration was determined by the method of Bradford (21) using bovine serum albumin as the standard. LY290181 was synthesized at the Eli Lilly Research Laboratories. The compound was prepared and stored in dimethyl sulfoxide as a 10 mM stock solution.

Determination of Steady-state Microtubule Polymer Mass—Tubulin pellets were thawed and centrifuged at 4 °C to remove any aggregated or denatured tubulin. Tubulin (13 \(\mu\)M) was mixed with S. purpuratus flagellar seeds (22) in 87 mM Pipes, 36 mM Mes, 1.4 mM MgCl₂, 1 mM EGTA, pH 6.8 (PMME buffer) containing 1 mM GTP and incubated at 37 °C in the absence or presence of different concentrations of LY290181 for 35 min to polymerize the microtubules. The microtubules were pelleted by centrifugation at 150,000 \(\times g\) for 1 h, and the microtubule pellets were solubilized in PMME buffer at 0 °C.

Video Microscopy—Tubulin was mixed with axoneme seeds and polymerized in PMME buffer containing 1 mM GTP in the presence or absence of LY290181. The seed concentration was adjusted to achieve 3–6 seeds per microscope field. After 35 min of incubation, samples of microtubule suspensions (2.5 \(\mu\)l) were prepared for video microscopy, and the dynamics of individual microtubules were recorded at 37 °C as described previously (22). The microtubules were observed for a maximum of 45 min after reaching steady state. Under the experimental...
conditions used, microtubule growth occurred predominantly at the plus ends of the seeds as determined by the growth rates, the number of microtubules that grew, and the relative lengths of the microtubules at the opposite ends of the seeds (5, 20, 22–25). Microtubule length changes were analyzed as described previously (22). We considered the microtubule to be in a growing phase if the microtubule increased in length by >0.2 μm at a rate >0.15 μm/min and in a shortening phase if the microtubule shortened in length by >0.2 μm at a rate >0.3 μm/min. Length changes equal to or less than 0.2 μm over the duration of six data points were considered as attenuation phases. We used the same tubulin preparation for all experiments, and an average of 25–30 microtubules was measured for each experimental condition.

We calculated the catastrophe frequency (a catastrophe is a transition from the growing or attenuated state to shortening (5)) by dividing the number of catastrophes by the sum of the total time spent in the growing plus attenuated states for all microtubules for a particular condition. The rescue frequency (a rescue is a transition from shortening to growing or attenuation, excluding new growth from a seed (5)) was calculated by dividing the total number of rescue events by the total time spent shortening for all microtubules for a particular condition.

Fluorescence Measurements—Fluorescence measurements were performed using a Perkin-Elmer LS50B spectrofluorometer equipped with a constant temperature water circulating bath. Spectra were taken by multiple scans, and buffer blank values were subtracted from all measurements. We used colchicine binding to tubulin using colchicine fluorescence induced upon the binding of colchicine to tubulin (26). 356 nm was used as the excitation wavelength and 435 nm as the emission wavelength. The excitation and emission band pass were 10 nm. All experiments were repeated at least twice.

The interaction of LY290181 with tubulin was monitored by its effects on the intrinsic tryptophan fluorescence of tubulin. When excited at 295 nm, tubulin displays a typical tryptophan emission spectrum with a maxima at 336 nm. We selected 295 nm as the excitation wavelength to specifically excite the tubulin tryptophan residues (27). Relative fluorescence intensities were measured at 25 °C, and buffer blank values were subtracted from all measurements. A tubulin concentration of 2 μM was used for all measurements. The inner filter effects were corrected empirically by measuring the change of fluorescence intensity of a tryptophan solution equivalent to the tubulin concentration in the presence of LY290181, and the corrected fluorescence intensities were used for all calculations (28). The fraction of binding sites (X) occupied by LY290181 was determined using Equation 1.

\[ X = \frac{(F_o - F_r)}{(F_o - F_m)} \]  

(Eq. 1)

where \( F_o \) is the fluorescence intensity of tubulin in the absence of LY290181; \( F_r \) is the corrected fluorescence intensity when the tubulin and LY290181 are in equilibrium, and \( F_m \) is the fluorescence intensity at the LY290181 concentration required to saturate the protein. The binding equilibrium constant was determined using Equation 2 (29).

\[ 1/X = 1/nK_o[P_o] + 1/L \]  

(Eq. 2)

\( [P_o] \) and \( [L] \) are the total protein and ligand concentration, respectively; \( K_o \) is the apparent equilibrium association constant, and \( n \) is the number of binding sites for LY290181. The experimental data were computer fitted to Equation 2 using MacCurvefit 1.0.1.

Vinblastine Binding to Tubulin—Vinblastine, 3 μM, containing a trace of [3H]vinblastine (specific activity 11 Ci/mmol) was mixed with tubulin (3 μM) and varying concentrations of LY290181 (0–20 μM). The mixtures were incubated at 37 °C for 40 min, and bound vinblastine was determined using a DE-81 filter paper assay as described previously (30).

Titration of Sulphydryl Groups—The sulphydryl-specific reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) complexes with thiol groups in tubulin and can be used to monitor conformational changes in tubulin due to ligand binding (31). Sulphydryl group modification kinetics were monitored colorimetrically at 412 nm (32). Tubulin (6.8 μM) was incubated with 25 μM LY290181 at 37 °C for 30 min and then 300 μM DTNB was added. After 1 h of incubation the number of modified sulphydryl groups was determined using a molar extinction coefficient of 12,000.

RESULTS

Inhibition of Microtubule Polymerization by LY290181—We first determined the ability of LY290181 (Fig. 1) to inhibit axonemal-seeded polymerization by measuring polymer mass using a sedimentation assay. As shown in Table I, LY290181 inhibited polymerization in a concentration-dependent manner; 50% inhibition occurred at 0.75 μM compound. The lowest concentration of LY290181 that detectably reduced the microtubule polymer mass was ~0.3 μM, and no reduction in polymer mass was detected at 0.1 μM LY290181 or less.

Effects of LY290181 on Microtubule Dynamics at Steady State—Microtubule growth occurred predominantly at the plus ends of the seeds as determined by the growth rates, the number of microtubules that grew, and the relative lengths of the microtubules at the opposite ends of the seeds (5, 20, 22–25). Several life history traces of microtubule length changes in the absence of LY290181 are shown in Fig. 2A. As previously documented, the microtubules alternated between phases of growing and shortening and also spent a small fraction of time in an attenuated state, neither growing nor shortening detectably. Addition of LY290181 suppressed the growing and shortening phases and increased the percentage of time that the microtubules spent in the attenuated state (Fig. 2B and C).

The actions of LY290181 on microtubule dynamics at steady state were determined quantitatively (Table I). LY290181 strongly suppressed the growing rates in a concentration-dependent manner. For example, the mean growing rate in the absence of LY290181 was 29 dimers/s, and addition of 0.1 μM LY290181 reduced the growing rate by 40%. At the highest concentration studied (0.9 μM), LY290181 reduced the mean growing rate by approximately 70% from 29 to 10.1 dimers/s. The average length microtubules grew per growing event was determined by dividing the summed growing lengths for all microtubules for a particular condition by the total number of growing events. As shown in Fig. 3, the length grown per growing event was strongly decreased by LY290181. For example, the mean growing length per growing event was reduced 70% at 0.9 μM LY290181. Thus, microtubules grew more slowly and grew shorter lengths in the presence of LY290181 than in its absence.

Similar to its effects on growing, LY290181 also strongly suppressed the rate and extent of shortening (Table I; Fig. 3). For example, the shortening rate was reduced 85%, and the mean length shortened during a shortening event was reduced by 79% at 0.9 μM LY290181. Interestingly, 0.1 μM LY290181 produced almost 50% inhibition of the growing and shortening rates, whereas it did not detectably alter the polymer mass (Table I). Thus, the kinetics of tubulin addition and loss at microtubule plus ends were substantially more sensitive to LY290181 than bulk phase polymerization.

At or near steady state, microtubules in vitro and in cells spend a considerable fraction of time in an attenuated or pause phase (19, 20, 22, 33). LY290181 significantly increased the percentage of time that the microtubules spent in the attenuated state (Table I). The transition frequencies among the growing, shortening, and attenuated states are considered to be important in the regulation of microtubule dynamics in cells.

![Fig. 1. Structure of LY290181 (2-Amino-4-(3-nitrophenyl)4H-naphtho(1,2-β)pyran-3-carbonitrile).](image)
LY290181 decreased the catastrophe frequency and increased the rescue frequency per unit time (Table II). We also calculated the number of transitions per μm of microtubule length grown and shortened (22, 36). The catastrophe frequency per μm was increased moderately by LY290181 (Table II). The increased catastrophe frequency per μm appeared to be due to a decrease in the growing rates. In contrast to the modest effects of LY290181 on the catastrophe frequency, LY290181 strongly increased the rescue frequency per μm. The modest increase in the catastrophe frequency per μm coupled with the dramatic increase in the rescue frequency per μm leads to extensive kinetic stabilization of microtubule dynamics.

**TABLE I**

| Parameter                        | 0          | 0.1        | 0.3        | 0.6        | 0.9        |
|---------------------------------|------------|------------|------------|------------|------------|
| Polymer mass (% of control)    | 100 ± 5.0  | 100 ± 13   | 83 ± 8.0   | 66 ± 20    | 40 ± 8.0   |
| Rate (dimer s⁻¹)                |            |            |            |            |            |
| Growing                         | 29.0 ± 12  | 17.5 ± 15.8| 13.2 ± 7.3 | 11.3 ± 9.0 | 10.1 ± 5.6 |
| Shortening                      | 468 ± 214  | 224 ± 318  | 166 ± 230  | 96 ± 197   | 70 ± 102   |
| Percent of total time           |            |            |            |            |            |
| Growing                         | 82.9       | 64.2       | 63.4       | 37.6       | 44.1       |
| Shortening                      | 12.1       | 7.4        | 8.3        | 5.8        | 6.2        |
| Attenuation                     | 5.0        | 28.4       | 28.3       | 56.6       | 49.7       |
| Dynamicity (dimers s⁻¹)         | 79         | 28         | 22         | 9.9        | 8.8        |

* a = S.E. Control dynamics data are the same as in Ref. 22. The control polymer mass was 0.3 mg/ml.
* b = S.D.

**FIG. 2.** Growing and shortening length changes of microtubules at their plus ends at steady state in the absence (A) and presence of 0.1 μM (B) and 0.9 μM (C) LY290181. The lengths of individual microtubules were measured from real time video tape recordings as described under “Experimental Procedures.”
Dynamicity is a parameter that reflects the overall dynamics of the microtubules (the total detectable tubulin dimer addition and loss at a microtubule end including the time spent in the attenuated state; 19, 20, 22). As shown in Table I, dynamicity was strongly suppressed by LY290181.

Binding of LY290181 to Tubulin—The binding of LY290181 to tubulin was investigated by measuring the effects of LY290181 on the intrinsic fluorescence intensity of tubulin. Tubulin (2 μM) was incubated with various concentrations of LY290181 (0–25 μM) at 25 °C for 50 min. As shown by the fluorescence quenching titration (Fig. 4A), LY290181 produced a concentration-dependent quenching of tubulin fluorescence. Analysis of these data (see “Experimental Procedures”) indicates that LY290181 binds to tubulin with an association constant of 3.8 ± 0.3 × 10^5 M^−1 and a stoichiometry of 0.98 ± 0.15 binding sites per tubulin dimer (Fig. 4B). The fluorescence quenching of tubulin by LY290181 did not significantly affect the position of the emission maximum wavelength, suggesting that binding of LY290181 does not modify the polarity in the vicinity of the tryptophan residues.

Inhibition of Tubulin-Bis-ANS Fluorescence by LY290181—The apolar molecule bis-(8-anilinonaphthalene-1-sulfonate) (bis-ANS) binds to tubulin and inhibits microtubule assembly substoichiometrically (37). Tubulin has one high affinity site (bis-ANS) binds to tubulin and inhibits microtubule assembly and multiple low affinity binding sites for bis-ANS (38, 39). The strong tubulin-bis-ANS-complex fluorescence and the extreme environmental sensitivity of bis-ANS make it a useful tool for probing conformational states of the tubulin dimer (37–40).

Tubulin (2 μM) was incubated with various concentrations of LY290181 for 50 min at 25 °C. Bis-ANS (20 μM) was added to the tubulin/LY290181 mixture, and incubation was continued for an additional 15 min. Fig. 5 shows that LY290181 produced a concentration-dependent quenching of bis-ANS fluorescence. Half-maximal quenching occurred at 4 μM LY290181. Furthermore, incubation of tubulin with bis-ANS prior to the addition of LY290181 also produced similar results (data not shown). The strong reduction in fluorescence of tubulin-bis-ANS complex by LY290181 could be due to the binding of bis-ANS and LY290181 to the same region of tubulin. Alternatively, LY290181 binding to tubulin may induce a conformational change in tubulin leading to the reduction in bis-ANS binding.

Competition between LY290181 and Colchicine—Colchicine and vinblastine bind to tubulin and microtubules and stabilize microtubule dynamics in a manner similar to that just described for LY290181 (11, 20, 25). Thus, we wanted to determine whether LY290181 binds to tubulin at the colchicine or vinblastine-binding site. Colchicine is not fluorescent, but it becomes strongly fluorescent when it binds to tubulin (26). Thus, we used the fluorescence of tubulin-colchicine (TC) complex to determine whether LY290181 competed with colchicine binding to tubulin. Twenty μM LY290181 inhibited development of TC complex fluorescence as shown in Fig. 6A (inset). The concentration dependence for inhibition of TC complex fluorescence by LY290181 is shown in Fig. 6A (circles). The results demonstrate that LY290181 is a potent inhibitor of colchicine binding with 7 μM LY290181 causing 50% reduction in binding.

There are two possible mechanisms by which LY290181 might inhibit TC complex fluorescence. First, LY290181 could compete with colchicine for the same binding site. Alternatively, LY290181 could bind to a site other than the colchicine site that prevents colchicine binding. To distinguish between these possibilities, TC complex was formed first, and TC complex fluorescence was then monitored upon addition of LY290181. As shown in Fig. 6A (triangles), LY290181 had no effect on the fluorescence of the preformed TC complex. These results demonstrate that LY290181 did not displace previously bound colchicine from TC complex.

LY290181 decreased the fluorescence of tubulin-bis-ANS complex in a concentration-dependent manner (Fig. 5), and thus we used tubulin-bis-ANS fluorescence as a probe for the binding of LY290181 to tubulin to determine whether LY290181 can bind to preformed TC complex. Bis-ANS binds to tubulin at a site or sites other than the colchicine binding site (39). Fig. 6B shows that incubation of TC complex with LY290181 decreased bis-ANS fluorescence, demonstrating that both LY290181 and colchicine bind to tubulin simultaneously. Therefore, LY290181 and colchicine bind to independent sites in tubulin. However, bound LY290181 could induce a conformational change in tubulin that prevented subsequent binding of colchicine.

LY290181 Does Not Bind to the Vinblastine-Binding Site in Tubulin—Several antimitotic compounds are known to inhibit the binding of vinblastine to tubulin, although there are no structural similarities among the compounds (14). Two ap-
proaches were used to determine whether LY290181 could bind to the vinblastine site. Since LY290181 reduced tubulin-bis-ANS fluorescence in a concentration-dependent manner (Fig. 5), we reasoned that if vinblastine could prevent the binding of LY290181 to tubulin, incubation of tubulin with vinblastine should eliminate the reduction in bis-ANS fluorescence. Thus, mixtures of vinblastine (0–30 μM) and 5 μM tubulin plus 10 μM bis-ANS were incubated at 37 °C for 15 min. Then 10 μM LY290181 was added to each mixture, and the incubation was continued for another 45 min. The results show that LY290181 strongly reduced tubulin-bis-ANS fluorescence and that addition of vinblastine did not affect the reduction at all concentrations of vinblastine examined (data not shown). We also determined whether LY290181 inhibited the binding of [3H]vinblastine to tubulin. Tubulin (3 μM) was incubated with 3 μM [3H]vinblastine in the absence and presence of LY290181, and vinblastine binding to tubulin was determined (see “Experimental Procedures”). We found that LY290181 did not inhibit vinblastine binding at the concentrations examined (5–20 μM, data not shown). Thus, we conclude that LY290181 does not bind at the vinblastine-binding site.

Kinetics of Chemical Modification of Tubulin Sulfhydryl Groups by DTNB—Modification of one to two sulfhydryl groups in tubulin can completely inhibit microtubule polymerization (41). Since these sulfhydryl groups appear to be located in regions of tubulin that are important for polymerization, the changes in the chemical reactivity of these residues could be a measure of conformational change. Thus, we determined the accessibility of the titratable cysteines to chemical modification by the sulfhydryl-specific reagent DTNB in the presence and absence of LY290181. Fig. 7 shows the reaction kinetics for cysteine titration in tubulin with DTNB at pH 6.8 in the absence and presence of 25 μM LY290181. LY290181 did not significantly affect the number of titratable cysteines at equilibrium; there were 12.2 ± 0.2 titratable sulfhydryl residues per tubulin dimer reacted in the absence of LY290181 and 11.2 ± 0.5 residues per tubulin dimer reacted in its presence. However, LY290181 did significantly reduce the initial rate of sulfhydryl residue modification (Fig. 7), indicating that binding of LY290181 to tubulin does induce a conformational change in the tubulin.

**Fig. 4.** Induction of fluorescence changes in tubulin by LY290181. A, tubulin (2 μM) was mixed with 0 (••••), 1 μM (---), 3 μM (-----), or 5 μM (——) LY290181, and emission spectra were measured after 50 min of incubation. The excitation wavelength was 295 nm. B shows protein fluorescence quenching titration following binding of LY290181 to tubulin. Fluorescence values at 336 nm were taken for the calculation. Data are representative of four different experiments.

**Fig. 5.** Quenching of tubulin bis-ANS-complex fluorescence by LY290181. Tubulin (2 μM) was incubated with the indicated concentrations of LY290181 for 50 min. Bis-ANS (20 μM) was then added to the incubation mixture. Excitation and emission wavelengths were 430 and 490 nm, respectively. Data are representative of two replicate experiments.

**Fig. 6.** Inhibition of colchicine binding to tubulin by LY290181. A, tubulin (5 μM) was first incubated with different concentrations of LY290181 (0–25 μM) at 37 °C for 20 min. Then 10 μM colchicine was added to all of the mixtures. Fluorescence (circles) was measured after 90 min of incubation at 37 °C (see “Experimental Procedures”). In a separate experiment, TC complex was first formed by incubating 5 μM tubulin with 10 μM colchicine at 37 °C for 90 min. Then LY290181 at different concentrations (0–25 μM) was added to the preformed TC complex, and fluorescence was determined after 60 min incubation (triangles). B, reduction of tubulin-bis-ANS fluorescence as a function of LY290181 concentration. TC complex was prepared as described in A and a fixed concentration (15 μM) of bis-ANS was added. Different concentrations of LY290181 (0–25 μM) were then added, and fluorescence was determined. The excitation and emission wavelengths were 430 and 490 nm, respectively.
LY290181 is a novel inhibitor of cell proliferation that acts at prometaphase of mitosis by an apparent action on microtubules. In the present study we found one LY290181 binding site per mol of tubulin with an association constant of 3.8 ± 0.3 × 10^5 M^−1. LY290181 did not bind to tubulin at the vinblastine or colchicine sites. While preincubation of LY290181 with tubulin inhibited the binding of colchicine to tubulin, LY290181 did not displace colchicine from its site once the colchicine was bound and the compound bound to the TC complex. Despite the differences in the molecular sites of action of all three compounds, LY290181 strongly inhibited microtubule growing and shortening dynamics at steady state in a manner similar to that of colchicine and vinblastine (11).

Mechanism of Stabilization of Microtubule Dynamics by LY290181—LY290181 strongly suppressed the rate of microtubule growth (Table I). At relatively high concentrations (>0.3 μM), LY290181 reduced the growing rate concomitant with a decrease in the polymer mass. However, low concentrations of LY290181 (0.1–0.3 μM) suppressed the growing rate without appreciably decreasing polymer mass.

The observed mean growing rate represents a balance between addition and dissociation events at the microtubule ends and can be written as shown in Equation 3.

\[ R_g = K_a[C] - K \]  

(Eq. 3)

\( R_g \) is the growing rate; \([C] \) is the soluble tubulin concentration; \( K_a \) is the dissociation rate constant; and \( K \) is the association rate constant. At 0.1 μM, LY290181 reduced the mean growing rate by ∼40% without changing [C]. Thus reduction of the observed growing rate by LY290181 could be due to either a reduction of \( K_a \), or an increase in \( K \). Since LY290181 strongly reduced the shortening rates (see below), it is unlikely to increase \( K_a \). Thus the reduction in the \( R_g \) would be due to reduction of \( K \). It is likely that there are multiple tubulin addition sites at a microtubule end (8, 42). Thus, \( K_a \) represents the combined individual rate constants for all sites (\( k_{a} \)), and \( K = k_{a} n \), where \( n \) is the number of addition sites. Thus, the reduction of \( K_a \) by LY290181 may be due either to reduction of \( k_{a} \), or \( n \) or to a combination of both. LY290181 might reduce \( n \), the number of addition sites, by steric hindrance or by inducing a conformational change at the addition site. Reduction of \( k_{a} \) by LY290181 could occur because the presence of the bulky LY290181 molecule might induce a strain in the microtubule lattice at the end that makes further tubulin addition energetically unfavorable. Thus, to relieve the strain, the newly added tubulin may adopt an incorrect geometry that makes future tubulin addition unfavorable in a propagated manner (reducing the \( k_{a} \)).

LY290181 also strongly stabilized microtubules by inhibiting the shortening rate (Table I), perhaps by strengthening lateral interactions between tubulin subunits between adjacent protofilaments. Such strengthening could be due to conformational changes induced by LY290181 binding to tubulin along the length of the microtubule or at its ends. Microtubules are thought to be composed of a labile tubulin-GDP core and a stable tubulin-GTP (or GDP-P) cap at their ends (6–8). Loss of the cap exposes the labile core and the microtubule rapidly disassembles. It is possible that LY290181-bound tubulin-GDP dissociates more slowly than unliganded tubulin-GDP because of a conformational change induced in the tubulin by LY290181 that mimics the conformation of the stabilizing tubulin-GTP cap. To reduce the shortening rate, LY290181 must bind transiently at or near the depolymerizing microtubule end or become incorporated at the ends as a tubulin-LY290181 complex.

The strong effects of LY290181 on the catastrophe and rescue frequencies indicate that LY290181 might directly act on the cap (Table II). LY290181 could reduce cap loss by reducing the rate of GTP hydrolysis or P release. Alternatively, LY290181 could stabilize microtubules ends by inducing a tubulin conformational change at the ends that does not involve the cap mechanism. Suppression of the shortening rate by LY290181 can also increase the rescue frequency indirectly by allowing more time during a shortening event for cap regain.

Effects of LY290181 on Polymer Mass in Relation to Suppression of Dynamics—LY290181 (0.9 μM) inhibited microtubule polymerization by 60%, but the microtubules that did form were highly stable kinetically. This apparent paradox can be explained by considering the linkage between ligand binding and tubulin conformational change at a microtubule end. The growth of a microtubule in the presence of LY290181 can be described as a balance among the bonding free energy for tubulin subunit addition to the end, the strain free energy due to alteration from optimal geometry caused by the bulky LY290181 molecule, and the free energy of stabilization due to a conformational change in tubulin induced by LY290181 binding. Microtubule growth will continue until the sum of the stabilizing free energies (bonding and conformational change) balance the strain free energy. After addition of a certain threshold number of LY290181 molecules to a microtubule end, it would be expected that the strain free energy will become greater than the sum of the stabilization free energies. At this point, microtubule growth becomes thermodynamically unfavorable, and the microtubule polymer mass will decrease even though the individual microtubules are kinetically more stable than microtubules in the absence of LY290181.

We found that microtubule dynamics were stabilized by LY290181 regardless of the final critical concentration achieved. In contrast to the depolymerization that occurred at relatively high LY290181 concentrations, low concentrations of LY290181 did not appreciably change the critical subunit concentration but did kinetically stabilize the microtubules (Table I). Thus, low concentrations of LY290181 can perturb kinetic events that are responsible for microtubule functions without depolymerizing the microtubules.

Implications for Cell Function—A large number of substances with chemically diverse structures have been identified that bind to tubulin in vitro and inhibit microtubule polymerization in vitro and in cells. The vinca alkaloid binding site and...
the colchicine binding site appear to be two important sites in tubulin, and many of the compounds thus far discovered bind to one of these sites (14). However, the drug taxol and other taxane analogs appear to bind at a third site, and drugs such as estramustine may bind to yet another region of tubulin. Thus, interference with normal microtubule function can occur by interactions of drugs with many sites in tubulin.

LY290181 binds to a novel site in tubulin. However, despite the differences in the molecular sites and mechanisms of action, LY290181, like vinblastine, colchicine, and taxol, stabilizes microtubule dynamics at low concentrations without significantly altering the microtubule polymer mass (20, 25, 43). These findings support the hypothesis that the mechanism underlying the ability of antimitotic compounds to inhibit cell proliferation and to kill tumor cells may be the kinetic stabilization of spindle microtubule dynamics (11). In view of the importance of microtubule dynamics in mitosis, it is reasonable to think that cells have available a sophisticated array of molecules and mechanisms to regulate microtubule dynamics and that compounds such as LY290181 are mimicking the actions of the natural regulators.

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