Perturbation of Microtubule Polymerization by Quercetin through Tubulin Binding: A Novel Mechanism of Its Antiproliferative Activity†

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ABSTRACT: The dietary flavonoid quercetin has a broad range of biological activities, including potent antitumor activity against several types of tumors. Recently, it has been shown that quercetin inhibits cancer cells proliferation by depleting cellular microtubules and perturbing cellular microtubule functions. However, the direct interactions of quercetin with tubulin and microtubules have not been examined so far. Here, we found that quercetin inhibited polymerization of microtubules and depolymerized microtubules made from purified tubulin in vitro. The binding of quercetin with tubulin was studied using quercetin fluorescence and intrinsic tryptophan fluorescence of tubulin. Quercetin bound to tubulin at a single site with a dissociation constant of 5−7 μM, and it specifically inhibited colchicine binding to tubulin but did not bind at the vinblastine site. In addition, quercetin perturbed the secondary structure of tubulin, and the binding of quercetin stimulated the intrinsic GTPase activity of soluble tubulin. Further, quercetin stabilized tubulin against decay and protected two cysteine residues of tubulin toward chemical modification by 5,5′-dithiobis-2-nitrobenzoic acid. Our data demonstrated that the binding of quercetin to tubulin induces conformational changes in tubulin and a mechanism through which quercetin could perturb microtubule polymerization dynamics has been proposed. The data suggest that quercetin inhibits cancer cells proliferation at least in part by perturbing microtubule functions through tubulin binding.

Microtubules are dynamic cytoskeletal polymers composed of $\alpha\beta$ tubulin heterodimers and are essential for many cellular activities, including maintenance of cell shape and structure, regulation of cell motility, intracellular transport, and cell growth and division (*1*−*2*). Normal cell division requires proper construction of the mitotic spindle apparatus, and microtubule dynamics play critical roles for the formation and functions of mitotic spindle apparatus (*3*−*5*). A large number of structurally unrelated compounds, many of which are obtained from natural products, perturb mitotic spindle functions and inhibit cell division at the metaphase/anaphase transition of mitosis by perturbing microtubule polymerization dynamics (*4*−*8*). Agents that target microtubules can be broadly divided into two classes; one group of compounds inhibits microtubule polymerization in vitro and depletes cellular microtubules while the second group of compounds increases microtubule polymerization (*4*−*9*). The first of these groups contains many families of compounds including *Vinca* alkaloids, colchicine, dolastatin and cryptophycins and many of these agents have distinct binding sites in tubulin *(4*−*6*, *8)*. The second group of compounds that enhances microtubule polymerization includes taxol, discodermolide and epothilones and these agents share overlapping binding sites in tubulin *(5*, *9)*. However, recent video microscopy studies demonstrated that both the depolymerizing and polymerizing classes of agents suppress microtubule dynamic instability without affecting the microtubule polymer level significantly (*4*−*5*, *8*, *10*−*16*). The clinical success of taxanes, *Vinca* alkaloids, and estramustine has prompted the search for new anticancer agents that target microtubules and several microtubule antagonists are currently undergoing clinical trials for cancer chemotherapy (*5*, *8*, *9*).

Flavonoids are of great current interest due to their antioxidative and possible anticancer activities (*17*−*28*). Recently, a series of flavones was found to be cytotoxic in the NCI 60-cell line assay and their cytotoxicity was linked with the inhibition of tubulin polymerization (*23*). Quercetin, a water-soluble plant pigment, is widely distributed in edible fruits and vegetables and has been demonstrated to inhibit proliferation of several types of cancer cells, including breast and prostrate cells in vitro (*24*, *27*, *29*). In addition, quercetin was found to have a broad spectrum of biological activities, including antihistamine, antiinflammatory, antioxidant, and anticoagulant properties (*17*, *22*, *25*, *26*). It has been shown that quercetin inhibits cell cycle progression at several stages of the cell cycle including G1/S, G2/M, and M (*18*, *26*, *27*, *29*). Several mechanisms, including inhibition of lactate transport, suppression of glycolysis and ATP production, inhibition of various tyrosine protein kinases, interference with ion pumps systems, and various signal transduction pathways and perturbation of microtubule network, have been cited for the antiproliferative activity of quercetin (*21*, *24*, *26*, *30*). Most recently, quercetin has been found to induce cell cycle arrest and triggers apoptosis in human breast cancer

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[1] Abbreviations: pipes, 1,4-piperazinediethanesulfonic acid; EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid; GTP, guanosine 5′-triphosphate; bis-ANS, bis-8-anilino-1-naphthalenesulfonic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DAPI, 4′, 6-diamidino-2-phenylindole; TC, tubulin−colchicine and buffer A, 25 mM pipes, pH 6.8, 3 mM MgSO4 and 1 mM EGTA.

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cells by transiently accumulating cells at the M phase of the cell cycle (*29*). However, the mechanism of action of quercetin is far from clear. An immunofluorescence study demonstrated that quercetin depolymerized microtubules of hormone refractory human prostate cancer cells (PC3) in culture (*24*). In addition, several flavonoids were shown to exert their antiproliferative activity by targeting microtubules through tubulin binding (*23*). These findings encouraged us to investigate the direct interaction of quercetin with tubulin and microtubules.

In this report, we have found that quercetin binds to tubulin at the colchicine site, stimulates GTPase activity of soluble tubulin, and perturbs microtubule polymerization by inducing conformational changes in tubulin. Our data suggest a novel mechanism of action for the antiproliferative activity of the natural antioxidant quercetin through tubulin binding. It is tempting to propose that quercetin could have potential clinical use in combination with known anticancer drugs that target microtubules for the treatment of various forms of cancer.

EXPERIMENTAL PROCEDURES

Materials. Quercetin, GTP, pipes, colchicine, and vinblastine were obtained from Sigma (St. Louis, MO). Bis-ANS was obtained from Molecular Probes (Eugene, OR). Phosphocellulose (P11) was from Whatman (Maidstone, England). All other reagents were of analytical grade.

Purification of Tubulin. Goat brain microtubule protein was isolated by two cycles of polymerization and depolymerization in the presence of 1 M glutamate and 10% v/v DMSO (*31*). Tubulin was purified from the microtubule protein by phosphocellulose chromatography (*32*) and stored at −80 °C. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (*33*).

Spectral Measurements. All fluorescence measurements were performed using a JASCO FP-6500 spectrofluorometer equipped with a constant temperature water-circulating bath. Spectra were taken by multiple scans, and buffer blank values were subtracted from all measurements. A 0.3 cm path length cuvette was used for all fluorescence measurements in order to minimize the inner filter effects. All absorbance measurements were performed in a JASCO V-530 UV−visible spectrophotometer using cuvette of 1 cm path length. The CD spectra were recorded in a JASCO J-810 spectropolarimeter at 25 °C using a 0.1 cm path length cuvette. The spectral data were acquired over the range of 250−210 nm.

Inhibition of Purified Tubulin Assembly by Quercetin. Tubulin (1.2 mg/mL) was mixed with different concentrations of quercetin (0−100 μM) in assembly buffer (25 mM pipes, pH 6.8, 3 mM MgSO4, 1 mM EGTA, 1 mM GTP, 1.0 M monosodium glutamate, pH 6.8), and the assembly reaction was initiated by incubating the sample at 37 °C. The rate and extent of the polymerization reaction were monitored by light scattering at 350 nm (*34*).

4′,6-Diamidino-2-phenylindole (DAPI) binds to double-stranded DNA, and the DAPI-DNA adduct fluoresces strongly (*35*). DAPI has been also shown to bind to both soluble tubulin dimers and microtubules (*36*). Interestingly, the relative fluorescence intensity of microtubule bound DAPI is significantly higher than the tubulin bound DAPI (*36*). Thus, we reasoned that if quercetin induces microtubule depolymerization, it would reduce the fluorescence intensity of microtubule-DAPI complex. Tubulin (12 μM) was polymerized along with 5 μM of DAPI under the conditions described above. Different concentrations of quercetin were added to the polymerized microtubule-DAPI complex, and the florescence signal of microtubule bound DAPI was used to monitor the effects of quercetin on preformed microtubules. The excitation and emission wavelengths were 390 and 450 nm, respectively.

Binding Measurements by Fluorometric Titration. A. Fluorescence of the Ligand. The increased quercetin fluorescence at 535 nm upon binding to tubulin was used to determine the affinity of tubulin and quercetin interaction. Initial experiments showed that the increase of quercetin fluorescence upon binding to tubulin attained saturation by 10 min of incubation at 25 °C. Tubulin (2 μM) was allowed to react with varying concentrations of quercetin (5−50 μM) in 25 mM pipes, pH 6.8, 3 mM MgSO4 and 1 mM EGTA (buffer A) at 25 °C for 30 min. The excitation and emission wavelength were 385 and 535 nm, respectively. Corrections due to the inner filter effect were done according to the formula $F = F_{obs}$ antilog $[(A_{ex} + A_{em})/2]$, where A_{ex} is the absorbance at the excitation wavelength and A_{em} is the absorbance at the emission wavelength (*37*). Quercetin has an extinction coefficient of 16 100 M^{-1} cm^{-1} at 385 nm. Binding parameters were determined using the relationship $1/X = K_d/[\text{free quercetin}] + 1$, where K_d corresponds to the dissociation constant and X is the fractional occupancy. The fractional occupancy (X) was determined using the relationship, $X = \Delta F/\Delta F_{max}$, where ΔF is the change in fluorescence intensity when tubulin and quercetin are in equilibrium, and ΔF_{max} is the value of maximum fluorescence change when tubulin is completely liganded with quercetin. ΔF_{max} was calculated by plotting $1/\Delta F$ versus $1/L$; using total ligand concentration as a first estimate of free ligand concentration and the calculation was repeated until there was no change in the value of the ΔF_{max} *(38, 39)*.

B. Quenching of the Protein Fluorescence. Tubulin (2 μM) was incubated with varying concentrations of quercetin (0−50 μM) at 25 °C for 40 min. The fluorescence measurements were performed using 295 as the excitation wavelength. We selected 295 nm as the excitation wavelength to specifically excite the tubulin tryptophan residues. When excited at 295 nm, tubulin displayed a typical emission spectrum with a maximum at 336 nm, and quercetin reduced the intrinsic fluorescence of tubulin. The apparent decrease in the fluorescence values in the presence of varying concentrations of quercetin were corrected for the inner filter effect as described previously. The fraction of binding sites (X) occupied by quercetin was determined using an equation $X = (F_o - F)/F_{max}$, where F_o is the fluorescence intensity of tubulin in the absence of quercetin, F is the corrected fluorescence intensity of tubulin in the presence of quercetin, and F_{max} is calculated from the plot of $1/(F_o - F)$ versus 1/[quercetin] graph and extrapolating 1/[quercetin] to zero. The dissociation constant (K_d) was determined using the relationship, $1/X = 1 + K_d/L_f$, where L_f represents free quercetin concentration, and $L_f = C - X[Y]$, where C is total concentration of quercetin and [Y] is the molar concentration of ligand-binding sites, assuming a single binding site per tubulin dimer (*11, 12*).

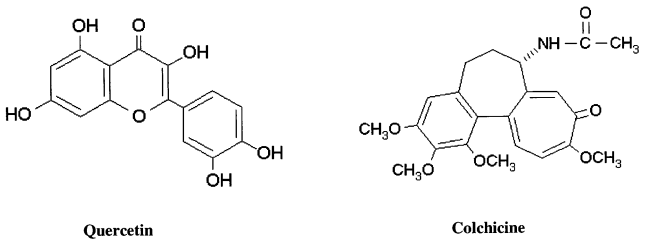

FIGURE 1: Structures of quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one] and colchicine.

Job Plot. The stoichiometry of binding was determined using the method of continuous variation *(40)*. Several mixtures of tubulin and quercetin were prepared by continuously varying concentrations of tubulin and quercetin in the mixture keeping the total concentration of quercetin plus tubulin constant at 10 μM. The reaction solutions were incubated for 40 min at 25 °C, and the fluorescence measurements were recorded using 385 nm as an excitation wavelength.

Colchicine Binding to Tubulin. Colchicine binding to tubulin was determined by using tubulin−colchicine fluorescence induced upon the binding of colchicine to tubulin (*41*, *42*). The excitation and emission wavelengths were 360 and 430 nm, respectively. The tubulin−colchicine complex was prepared by incubating 20 μM tubulin with 100 μM colchicine for 1 h at 37 °C, and the complex was purified by passing the tubulin−colchicine solution through a Sephadex G-25 size exclusion column at 4 °C *(43*, *44)*.

Titration of Sulfhydryl Groups. The sulfhydryl-specific reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) complexes with thiol groups in tubulin and the rate and extent of sulfhydryl groups modification could be monitored by measuring absorbance changes at 412 nm (*12*, *45*−*48*). Tubulin (3 μM) was incubated with 50 μM quercetin at 4 °C for 20 min, and then 200 μM DTNB was added. The number of sulfhydryl groups modified after 40 min of reaction was determined by using a molar extinction coefficient of 12 000 for TNB^- at 412 nm. The linear rate of sulfhydryl modifications was obtained by plotting $\ln[A_\infty/(A_\infty - A_t)]$ versus time, where A_∞ is the absorbance of TNB^- at saturation state and A_t is the absorbance at different times of the reaction.

Measurement of Quercetin-Induced GTPase Activity. We used a standard malachite green sodium molybdate assay to estimate the amount of inorganic phosphate released during the hydrolysis of GTP (*32*, *49*). Tubulin (6 μM) was incubated with different concentrations of quercetin for 30 min at 37 °C to form tubulin−quercetin complex, and the GTPase reaction was initiated by adding 1mM GTP and 6 mM $MgSO_4$ to the preformed tubulin−quercetin complex at 37 °C. The reaction was stopped at specific time intervals by addition of 70% perchloric acid. The quenched samples were stored on ice until all time points were collected. The samples were then incubated with malachite green at room temperature for 30 min, and then the absorbance at 650 nm were recorded. The background absorbance was subtracted from all readings.

RESULTS

Inhibition of Microtubule Polymerization by Quercetin. Quercetin (Figure 1) has been shown to inhibit human hormone refractory prostate carcinoma cells (PC3) proliferation by depleting the cellular microtubule network (*24*). Thus, we were interested to examine the effects of quercetin on microtubule polymerization behaviors in vitro. We first analyzed the ability of quercetin to inhibit polymerization of phosphocellulose-purified tubulin into microtubules in vitro. Purified tubulin (12 μM) was polymerized in the absence or presence of different concentrations of quercetin as described in the Experimental Procedures. Quercetin inhibited the rate and extent of tubulin polymerization in a concentration-dependent manner (Figure 2A). For example, 40 μM quercetin decreased the steady-state polymer level by 40%. The percentage inhibition of microtubule polymerization was calculated using the steady-state absorbance readings in the absence and presence of different concentrations of quercetin (Figure 2B), and 50% inhibition of microtubule polymerization (IC_{50}) occurred at quercetin concentration of 54 μM. Under similar conditions, 50% inhibition of microtubule polymerization occurred at 1.5 μM of colchicine.

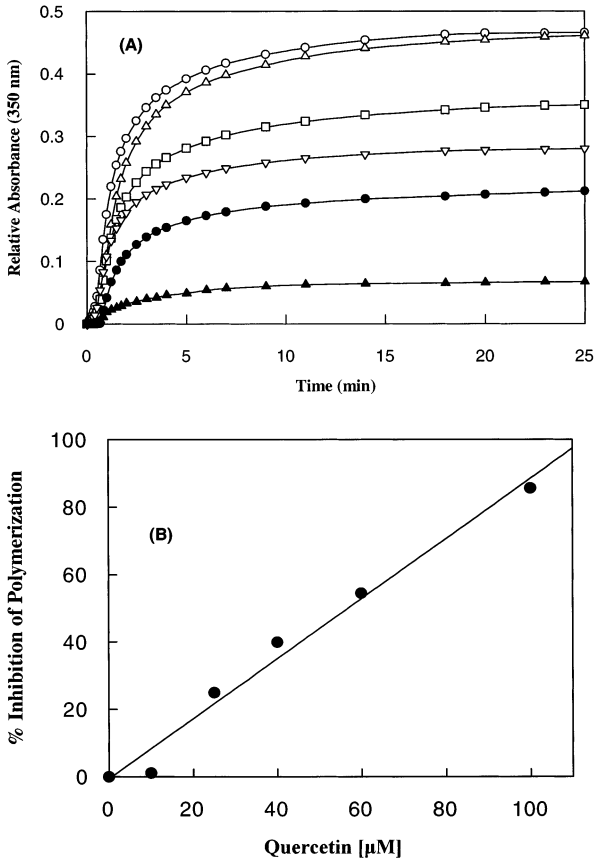

FIGURE 2: Inhibition of microtubules assembly by quercetin. (A) Polymerization of tubulin (12 μM) in the assembly buffer was measured in the absence (○) and in the presence of 10 (△), 25 (□), 40 (▽), 60 (●), or 100 μM (▲) quercetin. Microtubule assembly was monitored by measuring the increase in absorbance values at 350 nm with time. (B) Inhibition of tubulin polymerization at 25 min is plotted as a function of quercetin concentrations. Data are representative of three similar experiments.

Quercetin depolymerized PC3 cells microtubules (*24*). Thus, we also investigated whether quercetin could induce depolymerization of microtubules made from pure tubulin. The ability of quercetin to induce depolymerization of microtubules was analyzed by two different approaches. In the first method, we monitored the decrease in extrinsic

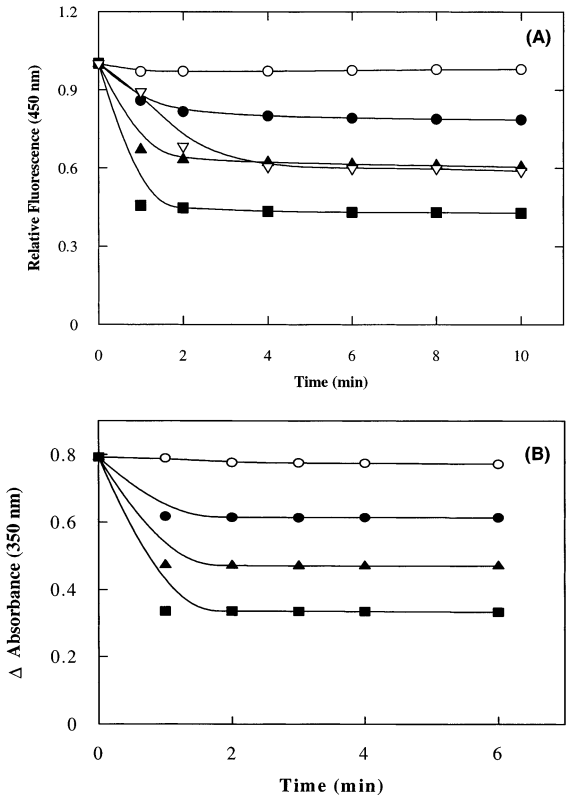

FIGURE 3: Disruptive effects of quercetin on preformed microtubules. (A) Microtubule depolymerization was monitored by measuring the decrease in DAPI fluorescence in the presence of different concentrations of quercetin. Tubulin (12 μM) was first polymerized in the assembly buffer with 5 μM DAPI for 20 min at 37 °C. Then 20 (●), 50 (▲), and 100 μM (■) quercetin, 20 μM vinblastine (▽), and 2% v/v DMSO (○) were added to the preformed microtubules. The excitation and emission wavelengths were 390 and 450 nm, respectively. (B) Depolymerization of preformed microtubules was monitored by light scattering at 350 nm in the absence (○) and presence of 20 (●), 50 (▲), and 100 μM (■) quercetin. Tubulin (12 μM) was first polymerized in the assembly buffer for 20 min at 37 °C, and different concentrations of quercetin were then added to the reaction mixtures.

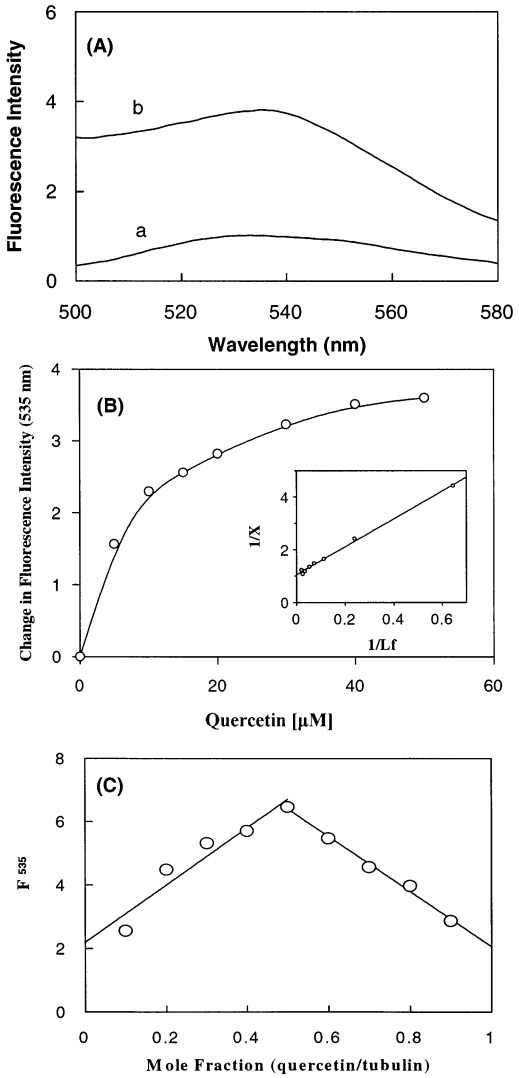

FIGURE 4: Quercetin binding to tubulin was measured by fluorescence spectroscopy. (A) Graph a shows the fluorescence spectrum of 5 μM quercetin in buffer A, and graph b shows the fluorescence spectrum of 5 μM quercetin in the presence of 5 μM tubulin. Spectra were taken after 30 min incubation at 25 °C. (B) Titration of 2 μM tubulin with the different concentrations (5.0−50 μM) of quercetin. The excitation wavelength was 385 nm. The inset shows a double reciprocal plot of quercetin binding to tubulin. (C) Job's plot of quercetin binding to tubulin. The concentrations of tubulin and quercetin were varied continuously keeping the total concentration of quercetin plus tubulin constant at 10 μM. The corrected fluorescence intensities at 535 nm were plotted against the mole fractions of quercetin.

fluorescence of DAPI−microtubule complex as a measure of microtubule depolymerization (*36*). Quercetin decreased the fluorescence of DAPI−microtubule complex in a concentration-dependent fashion, suggesting that quercetin induced microtubule depolymerization (Figure 3). Control experiments using vinblastine as a positive control and DMSO as a negative control were performed to demonstrate that the observed decreases in the fluorescence intensities upon addition of quercetin were not due to any artifact of the measurements but due to depolymerization of preformed microtubules (Figure 3A). As expected, vinblastine (20 μM) significantly reduced the DAPI−microtubule complex fluorescence, whereas DMSO had no detectable effect on the complex fluorescence.

In the second approach, we used the change in optical density at 350 nm as a measure of polymer mass (*34*). Tubulin (12 μM) was polymerized in the presence of 1 M glutamate for 20 min at 37 °C, and different concentrations of quercetin were added to the preformed microtubule suspensions. Quercetin induced rapid depolymerization of microtubules in a concentration-dependent fashion, as indicated by the decrease in the absorbance values at 350 nm (Figure 3B). For example, 50 μM quercetin reduced the polymer level by 41%, which was similar to the depolymerization measured using DAPI fluorescence (Figures 3A,B).

Binding of Quercetin to Tubulin. Quercetin has weak fluorescence in neutral aqueous buffer (Figure 4A), with maxima at 535 nm. When quercetin was mixed with tubulin in a 1:1 molar ratio, the fluorescence intensity of quercetin increased markedly (Figure 4A). For example, the fluorescence intensity of 5 μM quercetin was increased ~4-fold in the presence of equimolar concentration of tubulin; however, the emission spectrum of the quercetin did not show any significant shift upon binding to tubulin. The Figure 4B shows the titration curve of a constant amount of tubulin with various concentrations of quercetin. The binding data were analyzed as described in the Experimental Procedures, and the analysis of the data yielded a linear plot with a

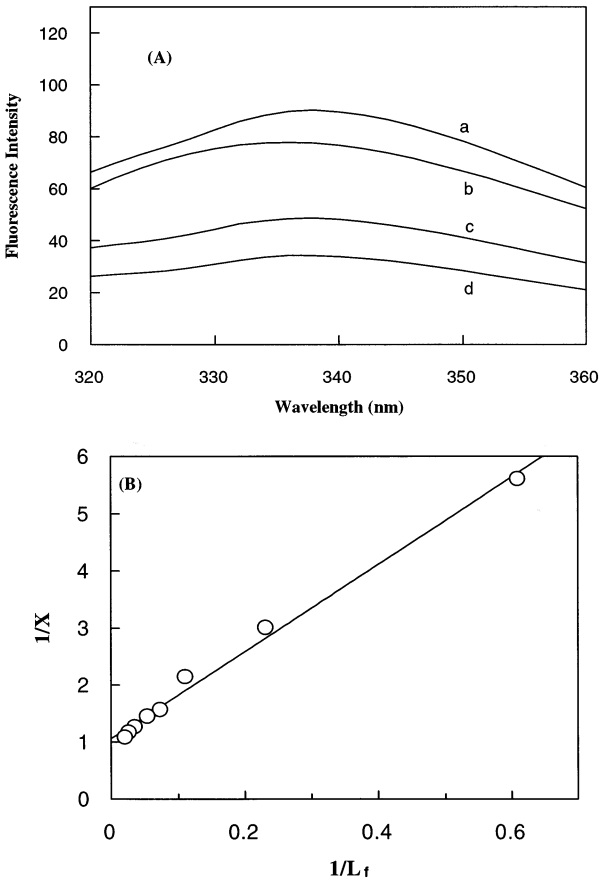

FIGURE 5: Effects of quercetin on the intrinsic tryptophan fluorescence of tubulin. (A) Tubulin (2 μM) was mixed with varying concentrations of quercetin from 0 (a), 5 (b), 20 (c) or 50 μM (d) quercetin in buffer A, and emission spectra were recorded after 40 min of incubation at 25 °C. (B) Double reciprocal plot of binding of quercetin to tubulin. Data are representative of three identical experiments.

dissociation constant of 6.5 ± 1.2 μM (Figure 4B inset). The stoichiometry of binding was determined using the method of continuous variation. The Job plot yielded a single binding site for quercetin in tubulin (Figure 4C). Taking together, these data suggested that quercetin binds to tubulin at a single site.

The binding of quercetin to tubulin was also investigated by measuring the effects of quercetin on the intrinsic tryptophan fluorescence of tubulin. As shown in Figure 5A, quercetin reduced the intrinsic tryptophan fluorescence of tubulin in a concentration-dependent fashion. However, quercetin did not affect the position of the emission maximum wavelength, suggesting that binding of quercetin did not alter the polarity of the immediate environment of tryptophan residues in tubulin. The double reciprocal plot of the binding data (Figure 5B) yielded a dissociation constant of 5.30 ± 1.5 μM, which is in excellent agreement with the K_d obtained by the ligand fluorescence titration.

Competition between Quercetin and Colchicine. Many structurally unrelated natural and synthetic compounds that inhibit microtubule polymerization bind either to the vinblastine-binding site or to the colchicine-binding site in tubulin (*4*−*6*, *8*). Like vinblastine and colchicine, quercetin also inhibited tubulin polymerization and induced depolymerization of preformed microtubules (Figures 2A and 3). Thus, we examined whether quercetin binds at the colchicine

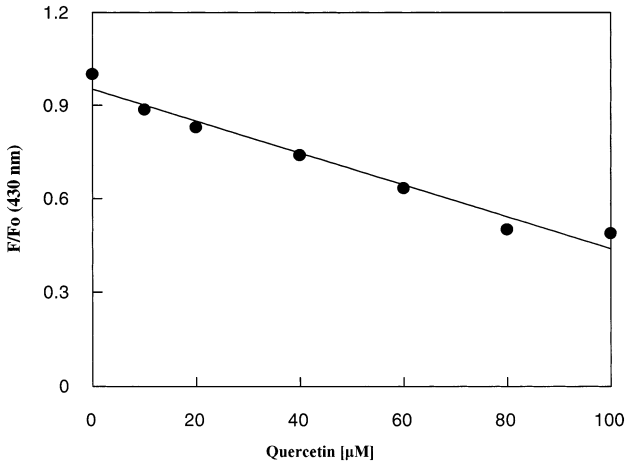

FIGURE 6: Quercetin inhibited the binding of colchicine to tubulin. Tubulin (6 μM) was incubated with different concentrations of quercetin (0−100 μM) at 37 °C for 20 min to form tubulin−quercetin complex. Colchicine (6 μM) was added to all of the mixtures and incubated at 37 °C for 60 min. Fluorescence spectra were recorded using 360 nm as an excitation wavelength.

or vinblastine site of soluble tubulin. Colchicine is weakly fluorescent in aqueous solution, but it becomes strongly fluorescent when it binds to tubulin (*41*). We used the fluorescence of tubulin−colchicine complex to determine whether quercetin shares binding site with colchicine in tubulin. The extent of colchicine binding at various concentrations of quercetin is shown in Figure 6. Quercetin inhibited colchicine binding to tubulin in a concentration-dependent fashion. For example, 40 μM quercetin inhibited colchicine binding to tubulin by 30%.

Colchicine binds to tubulin tightly and the dissociation of colchicine from tubulin−colchicine (TC) complex is a slow process (*50*). Podophyllotoxin, a compound that binds to the colchicine site on tubulin, has been shown to displace colchicine from TC complex albeit slowly (*43*, *44*). For example, incubation of preformed TC complex with 100 μM podophyllotoxin at 35 °C for 3 h reduced the colchicine binding by ~20% *(43)*. We used similar strategy to know whether quercetin could displace colchicine from the preformed TC complex. Incubation of TC complex (2 μM) with 5, 25, and 50 μM of quercetin for 1 h at 37 °C reduced the preformed TC complex fluorescence by 10%, 25%, and 32%, respectively, indicating that quercetin could displace colchicine from TC complex.

TC complex fluoresces with a maximum at 430 nm (*12*, *41*), whereas quercetin−tubulin complex fluoresces with a maximum at 535 nm (Figure 4A). Thus, we followed the quercetin binding to tubulin in the absence or presence of different concentrations of colchicine.

Quercetin binding to tubulin was inhibited by 14%, 28%, and 40% in the presence of 5, 30, and 50 μM of colchicine, respectively. Taking together, these data suggested that quercetin-binding site in tubulin overlaps with the colchicine-binding site.

Quercetin Does Not Bind to the Vinblastine-Binding Site in Tubulin. We used the quercetin−tubulin complex fluorescence to determine whether quercetin could bind to the vinblastine site. We reasoned that if vinblastine could inhibit the binding of quercetin to tubulin, incubation of tubulin with vinblastine should decrease the development of quercetin−

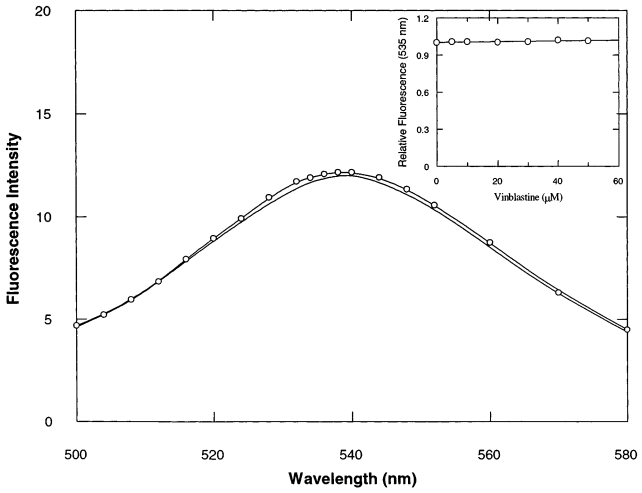

FIGURE 7: Effects of vinblastine on the binding of quercetin to tubulin. Tubulin (2 μM) was incubated in the absence (—) and presence (○) of vinblastine (5−50 μM) for 20 min to form tubulin-vinblastine complex. Thirty micromolar quercetin was added to the reaction mixtures and incubated for 30 min at 25 °C. Excitation and emission wavelengths were 385 and 535 nm, respectively.

tubulin complex fluorescence. Tubulin (2 μM) was incubated in the absence and presence of vinblastine (0−50 μM) to form tubulin-vinblastine complex, and then 30 μM of quercetin was added to the reaction mixtures. Vinblastine did not affect the tubulin−quercetin complex fluorescence, indicating that quercetin-binding site was different from the vinblastine-binding site (Figure 7).

Inhibition of Tubulin−Bis-ANS Fluorescence by Quercetin. The hydrophobic molecule bis-8-anilinonaphthalene-1-sulfonate (bis-ANS) binds to tubulin and inhibits microtubule assembly substoichiometrically (*51*). Tubulin contains one high affinity-binding site and multiple low-affinity binding sites for bis-ANS (*51*−*53*). The high-affinity bis-ANS binding site in tubulin does not overlap with the binding sites for colchicine, podophyllotoxin, vinblastine, or maytansine (*51*−*53*). The extreme environmental sensitivity of bis-ANS makes it a useful probe for examining conformational states of the tubulin dimer. Bis-ANS (25 μM) was added to preformed tubulin−quercetin complex, and the bis-ANS fluorescence was used to monitor the effect of quercetin on binding of bis-ANS to tubulin. Quercetin produced a concentration-dependent quenching of bis-ANS fluorescence (Figure 8A). For example, quercetin (20 μM) reduced the tubulin−bis-ANS fluorescence by 40%. Furthermore, incubation of tubulin with bis-ANS prior to the addition of quercetin also produced similar results (data not shown). The reduction in fluorescence of tubulin−bis-ANS complex fluorescence by quercetin could be due to the binding of bis-ANS and quercetin to the same region of tubulin. Alternatively, quercetin binding to tubulin may induce a conformational change in tubulin leading to the reduction in the bis-ANS binding.

Effects of Quercetin on the Tubulin Decay. One of the prominent characteristics of tubulin in solution is that it rapidly loses its colchicine binding activity and its ability to polymerize through a process called decaying of tubulin (*54*). Because the intensity of tubulin−bis-ANS fluorescence increases with the decaying of tubulin, it has been used extensively to probe the affects of tubulin binding agents on its decay process (*53*−*56*). Quercetin suppressed the time-dependent increase in tubulin−bis-ANS fluorescence, indicating that quercetin stabilizes tubulin against decay (Figure 8B).

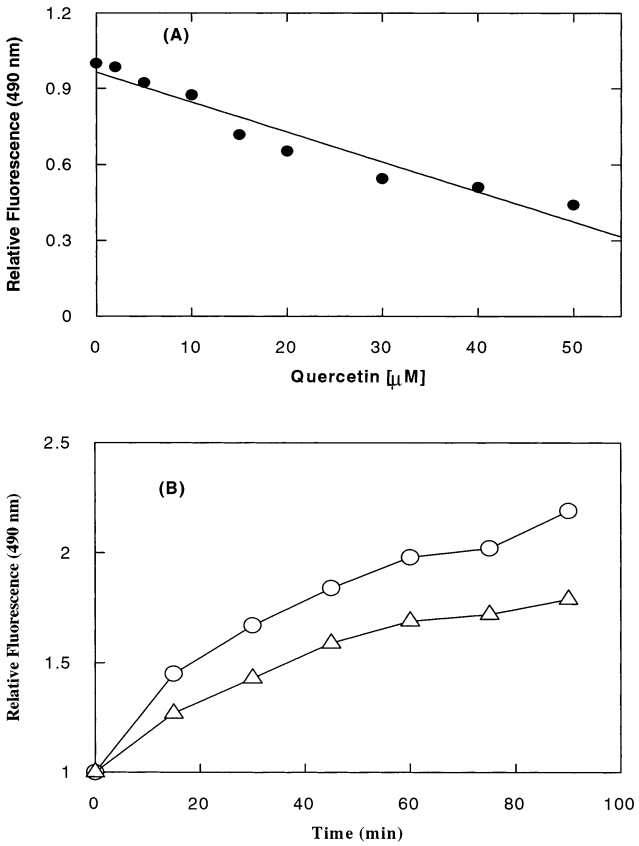

FIGURE 8: (A) Quenching of tubulin−bis-ANS complex fluorescence by quercetin. Tubulin (2 μM) was incubated with the 0−50 μM concentrations of quercetin for 40 min to form tubulin−quercetin complex. Bis-ANS (25 μM) was added to the complex, and fluorescence measurements were recorded after 20 min of incubation at 25 °C. Excitation and emission wavelengths were 430 and 490 nm, respectively. Data are representative of two replicate experiments. (B) Effects of quercetin on tubulin decay. Tubulin (2 μM) was incubated at 37 °C for different lengths of time (0−90 min) in the absence (○) and presence of 50 μM quercetin (△). At a specific time intervals, 25 μM bis-ANS was added to the reaction mixtures and incubated for an additional 15 min. The time-dependent increase in tubulin−bis-ANS fluorescence was monitored by measuring fluorescence intensity at 490 nm.

Quercetin Stimulates GTPase Activity of Soluble Tubulin. Soluble tubulin has a very low intrinsic GTPase activity, but some of the colchicine site ligands have shown to increase the GTPase activity of soluble tubulin (*56*−*58*). Quercetin has no apparent structural similarities with colchicine (Figure 1); however, it binds to the colchicine site in tubulin (Figure 6) and inhibits microtubule polymerization (Figure 2). Thus, we wanted to know whether quercetin could stimulate the GTPase activity of tubulin dimers. The kinetics of stimulation of GTPase activity of soluble tubulin by quercetin is shown in Figure 9. Quercetin stimulated GTPase activity in a concentration-dependent manner (Figure 9 inset). The data indicated that quercetin binding induces conformational changes in tubulin similar to colchicine site agents, which stimulate GTPase activity of tubulin.

Kinetics of Chemical Modification of Tubulin Cysteine Groups by DTNB. Modification of one to two cysteine groups in tubulin can completely inhibit microtubule polymerization

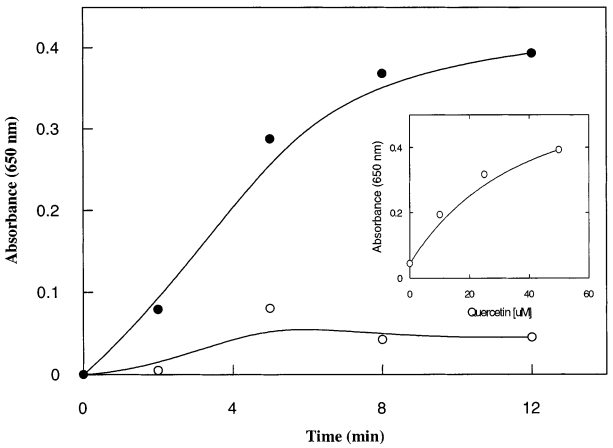

FIGURE 9: GTP hydrolysis by tubulin in the absence (○) or presence of 50 μM quercetin (●). Tubulin (6 μM) was preincubated with different concentrations (0−50) of quercetin, and the quercetin-induced GTPase activity of soluble tubulin was determined at 37 °C as described in the Experimental Procedures. The inset shows the effect of quercetin concentrations on the tubulin GTPase activity after incubating the reaction mixtures for 12 min.

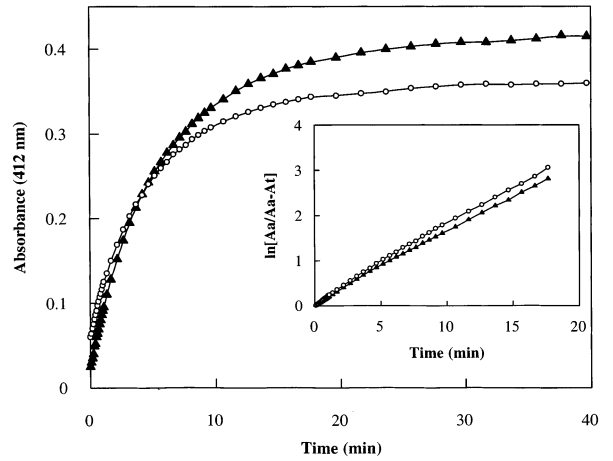

FIGURE 10: Chemical modifications of cysteine residues of tubulin by DTNB in the absence (▲) or in the presence of 50 μM quercetin (○) were monitored at 412 nm as described in the Experimental Procedures. The inset shows the pseudo first-order plot of the sulfhydryl modification kinetics in the absence (▲) and presence (○) of quercetin. Data were taken from one of the three similar experiments.

(*46*, *47*). These cysteine groups appear to be located in regions of tubulin that are important for polymerization, and modifications of these residues affect the polymerization of tubulin. The changes in the chemical reactivity of these residues upon ligand binding could be a measure of conformational change in tubulin due to ligand binding. Thus, we determined the accessibility of the cysteine residues to chemical modification by the sulfhydryl specific reagents DTNB in the presence and absence of quercetin. Figure 10 shows the reaction kinetics of cysteine modification with DTNB at 37 °C in the absence and presence of 50 μM quercetin. Quercetin reduced the average number of DTNB accessible cysteine residues per tubulin dimer by 2; there were 10.8 ± 0.10 and 8.6 ± 0.15 cysteine residues per tubulin dimer accessible to DTNB in the absence and presence of 50 μM quercetin (Figure 10). However, the pseudo-first-order plots of the data showed that quercetin did not affect the average rate of modification of cysteine groups of tubulin by DTNB (Figure 10, inset).

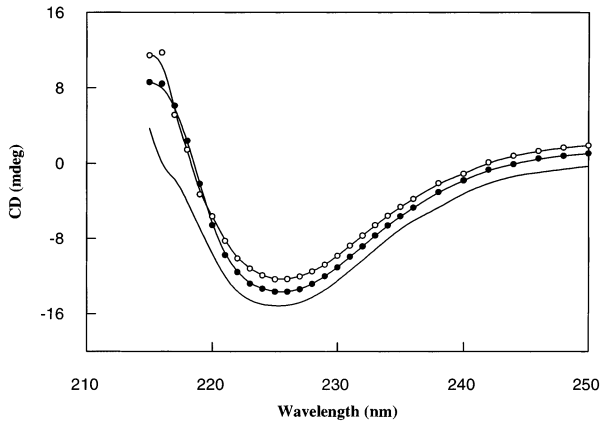

FIGURE 11: Far-UV CD spectra of 3 μM tubulin in the absence (—) and presence of 25 μM (●) and 50 μM (○) quercetin in 10 mM phosphate buffer (pH, 6.8) at 25 °C.

Effects of Quercetin on the CD Spectrum of Tubulin. Quercetin induced GTPase activity in tubulin, decreased tryptophan fluorescence, and stabilized tubulin against decay indicating that quercetin induces conformational changes in tubulin. The effect of quercetin on the secondary structure of tubulin was further examined by circular dichroism spectroscopy. As shown in Figure 11, quercetin altered the far-UV CD spectrum of tubulin indicating that quercetin binding altered the helical content in tubulin.

DISCUSSION

Quercetin, an edible plant flavonoid, has shown to inhibit proliferation of several cancer cell types (*18*−*28*); however, its mechanism of action is not clear. In this report, we found that quercetin inhibited the polymerization of purified tubulin in vitro, depolymerized preformed microtubules. and bound to tubulin at the colchicine-binding site with a dissociation constant of 5−7 μM. Binding of quercetin to tubulin induced changes in the secondary structure of tubulin, increased the intrinsic GTP-ase activity of soluble tubulin, reduced average number of accessible cysteine residues toward chemical modification by DTNB, and prevented tubulin decay. Using immunofluorescence microscopy and western blot analysis, Takagi et al. (1998) (*24*) reported that quercetin depolymerized microtubules of prostate cancer cells in culture. The studies presented here, together with those of Takagi et al. (1998) (*24*), strongly indicate that the natural flavonoid quercetin inhibits cancer cells proliferation by perturbing microtubule assembly.

Binding of Quercetin to Tubulin. The binding of quercetin with tubulin increased the fluorescence of quercetin, which provided a tool to probe the interaction of quercetin with tubulin (Figure 4A). Colchicine binding to tubulin also increases its fluorescence intensity, and several mechanisms, including immobilization of colchicine, restricted rotation, and changes in the electronic environment, have been reported to explain the increase of colchicine fluorescence (*41*, *42*, *59*, *60*). On similar lines, the enhancement of quercetin fluorescence upon binding to tubulin may be due to a restriction of the free rotation of quercetin in the quercetin−tubulin complex or changes in the electronic environment of the quercetin in the quercetin-tubulin complex. The dissociation constant for quercetin binding to tubulin was calculated to be 6.5 ± 1.2 μM using a double

reciprocal plot (Figure 4B inset). Quercetin binding decreased the intrinsic tryptophan fluorescence of tubulin indicating that its binding perturbed tubulin conformation in the vicinity of tryptophan residues. However, tubulin contains multiple tryptophan residues and it is not possible to discern which tryptophan residues are perturbed by quercetin. The reduction in tubulin fluorescence upon quercetin binding to tubulin also produced a dissociation constant of 5.30 ± 1.5 μM, which is in excellent agreement with the K_d obtained by the ligand fluorescence titration (Figure 5B).

A large number of antiproliferative agents with chemically diverse structures have been identified that inhibit microtubule polymerization and bind to tubulin in vitro. The *Vinca* alkaloid site and the colchicine site are two important drug-binding sites in soluble tubulin, and many of the microtubule depolymerizing agents bind to one of these sites. Vinblastine did not inhibit the binding of quercetin to tubulin, suggesting that quercetin did not bind to the vinblastine site in tubulin. However, quercetin inhibited the binding of colchicine to tubulin in a concentration-dependent manner (Figure 6). It is possible that quercetin inhibited colchicine binding by inducing conformational changes in tubulin rather than directly binding at the colchicine site. To distinguish between the possibilities, we investigated whether colchicine could inhibit the binding of quercetin to tubulin. Our data demonstrated that colchicine potently inhibited the binding of quercetin to tubulin. Taking together, the findings demonstrated that quercetin binds to tubulin at the colchicine site.

Consequences of Quercetin Binding to Tubulin. The modification of one or more cysteine residues of tubulin exerted inhibitory effects on the ability of tubulin to polymerize into microtubules (*46*−*47*). In addition, the cysteine residues of tubulin are thought to be involved in the binding of antimitotic drugs (*47*). For example, the binding of colchicine to tubulin was shown to involve Cys-239 and Cys-354 of β-tubulin (*61)*. The alteration in the rate and extent of the chemical modification of cysteine residues by tubulin-targeted drugs has been exploited to understand the interaction of drug molecules with tubulin. Quercetin binding reduced the average number of accessible cysteine residues from 10.8 ± 0.10 to 8.6 ± 0.15 per tubulin dimer without perturbing the rate of cysteine modification (Figure 10). The data indicate that the binding of quercetin to tubulin masked these residues for chemical modification, and these cysteine residues were possibly involved in the binding of quercetin to tubulin. However, the cysteine residues are distributed through out the protein, and it is quite possible that cysteine residues affected by quercetin are not located at the quercetin-binding site on tubulin but located elsewhere on the molecule, and quercetin-induced conformational change(s) prevented the chemical modification of these residues. Similar to quercetin, colchicine also protects 1.4−2 cysteine residues from alkylating agents (*47*, *48*). However, unlike quercetin, colchicine binding to tubulin causes a strong reduction in the reactivity of the cysteine residues (*47*, *48*), indicating that although colchicine and quercetin bind to the same site on tubulin, they exert differential effects on tubulin structure.

Tubulin loses its colchicine binding ability and its ability to polymerize into microtubules with time through an ill-defined process called decay (*54*). Several microtubule-depolymerizing agents, including vinblastine, cryptophycin-52, and colchicine, have shown to suppress the rate of decaying of tubulin (*53*, *55*, *56*). Quercetin binding to tubulin suppressed the rate of tubulin decay (Figure 8B), indicating that quercetin binding induced conformational change in tubulin. Further, quercetin perturbed the far-UV CD spectrum of tubulin, indicating that the binding of quercetin to tubulin perturbed the helical structure of tubulin (Figure 11). Soluble tubulin has extremely low GTPase activity and polymerization of tubulin dimers into microtubules stimulated the GTPase activity at the microtubule ends. The binding of quercetin to tubulin increased the GTPase activity of soluble tubulin (Figure 9). The data supports the idea that quercetin binding produces conformational change in tubulin, which is similar to conformational change that occurs in tubulin at the ends of microtubules during its polymerization.

Mechanism of Inhibition of Microtubule Polymerization. The data presented in this report suggested that the inhibitory effects of quercetin on microtubule polymerization were due to quercetin-induced conformational changes in tubulin. There are several possible mechanisms through which quercetin could inhibit microtubule polymerization. One of the possibilities is that quercetin exerts its inhibitory effects on microtubule polymerization by sequestering soluble tubulin, which makes tubulin incompetent for polymerization. The second possibility is that the quercetin−tubulin complex copolymerizes with free tubulin and incorporation of quercetin−tubulin complex in the microtubule lattice, along with tubulin distorts the spatial geometry of the microtubule lattice. The perturbed arrangement of the microtubule lattice decreased the polymer level, as described previously for the tubulin−colchicine complex (*62*). However, we could not rule out the possibility that quercetin can exert its activities through end poisoning mechanism as reported previously for colchicine (*4*, *5*, *63*). The presence of quercetin molecules in the form of quercetin−tubulin complex at the microtubule ends can modify the ends in such a way that further tubulin addition becomes energetically unfavorable. Alternatively, quercetin is a bulky molecule, and it could inhibit tubulin polymerization at microtubule ends by imposing steric hindrance due to its size.

Interestingly, quercetin bound to tubulin with a dissociation constant of 5−7 μM, and 50% inhibition of microtubule polymerization occurred at 54 μM of quercetin, indicating that most of the tubulin molecules will be complexed with quercetin at the inhibitory concentration of quercetin. Thus, a tubulin−quercetin complex gets incorporated at the microtubule ends, like a TC complex; however, its effects on tubulin polymerization are relatively weaker than those of colchicine. In other words, a quercetin−tubulin complex can form copolymers with tubulin−GTP efficiently, and the copolymerization of quercetin−tubulin complex into microtubules is a favored mechanism for the inhibition of microtubule polymerization.

Implications for Cell Function. Quercetin has been shown to inhibit cell proliferation by depolymerizing cellular microtubules (*24*). In the present study, we found that quercetin inhibited microtubule polymerization through tubulin binding. Thus, the antiproliferative activity of quercetin could be related to its ability to bind to tubulin and to perturb microtubule polymerization dynamics. Antimitotic compounds commonly perturb mitotic spindle function by interfering with microtubule dynamics, and this effect is

reflected in their abilities to modulate the polymerization of microtubules in vitro. Like other microtubule depolymerizing agents, quercetin inhibited the polymerization of tubulin into microtubules in vitro, suggesting that the target of its action is contained in these preparations. The drug value of colchicine and its analogues has been limited because of their severe harmful side effects (5). Quercetin, a natural flavonoid, is associated with many dietary products and has potent antioxidant activity, indicating that quercetin may have a low level of harmful side effects compared to some of the known microtubule poisons. Since quercetin exerts its antiproliferative activity at least in part through perturbing microtubule polymerization behavior, it could have potential clinical use in combination with known antimitotic anticancer agents such as taxol, vinblastine, and estramustine for the treatment of various forms of cancer.

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