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Griseofulvin: A Novel Interaction with Bovine Brain Tubulin

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ABSTRACT. Griseofulvin is an anti-fungal drug whose mechanism of action is directed against microtubules. Although it inhibits the assembly of mammalian brain tubulin, its binding to tubulin has not been directly measured successfully. We have examined the interaction of griseofulvin with tubulin fluorometrically by measuring the quenching of tubulin tryptophan fluorescence by griseofulvin. From Scatchard analysis, we found that griseofulvin bound to tubulin at one class of binding site with an affinity constant of $1.26 \pm 0.19 \times 10^4\ M^{-1}$, and the binding was largely reversible. Griseofulvin caused a major change in the conformation of tubulin in that it increased the sulfhydryl titer of tubulin approximately 2-fold. The drug affected both the α and β subunits of tubulin equally. Interestingly, griseofulvin did not increase the sulfhydryl titer of the tubulin–colchicine complex although the binding site of griseofulvin was distinctly different from that of colchicine. The change of conformation of tubulin upon interaction with griseofulvin did not affect the exposure of hydrophobic areas on tubulin as shown by binding of bis-5,5′-[8(*N*-phenyl)aminonapthalene-1-sulfonic acid] (BisANS). Even in combination with colchicine, griseofulvin had very little effect on BisANS binding to tubulin. Thus, griseofulvin appears to interact with tubulin in a manner that is very different from that of many other tubulin ligands. BIOCHEM PHARMACOL 51;7:903–909, 1996.

KEY WORDS. tubulin; griseofulvin; alkylation; hydrophobic areas; fluorescence; sulfhydryl groups

Microtubules are long ubiquitous cellular organelles that participate in a variety of cellular processes, including mitosis, intracellular transport, and cellular motility [1]. The major component of microtubules is tubulin, which is a heterodimeric protein having two 50 kDa subunits designated α and β [2]. The ligands of tubulin have been useful probes for studying the conformation of the tubulin molecule. Much is known about the mechanism of the binding of some of these drugs, such as colchicine, vinblastine, and others [3–7]. However, one drug whose interaction with tubulin is poorly understood is griseofulvin, a mold metabolite produced by *Penicillium*, which has antimitotic activity and interferes with the assembly of microtubules *in vivo* and *in vitro* [8], although it has also been reported that griseofulvin does not disrupt microtubules [9]. Griseofulvin has been used widely as an anti-fungal drug particularly against dermatophytes [10]. There are conflicting reports in the literature, suggesting that griseofulvin binds either to tubulin directly [11, 12] or to one or more of the MAPs† [13], which are known to interact with microtubules both *in vivo* [14] and *in vitro* [15]. Analysis of peptide maps obtained by digesting tubulin in the presence of various ligands showed that the pattern seen with griseofulvin is distinctly different from that generated in the presence of colchicine, vinblastine, taxol, and podophyllotoxin [16]. To date, the binding of griseofulvin to tubulin has not been directly measured successfully.

We devised a fluorometric assay and re-examined the interaction of griseofulvin with tubulin and found that it binds to tubulin at one class of binding site with a K_a of $1.26 \pm 0.19 \times 10^4\ M^{-1}$. We also found that griseofulvin does not inhibit the binding of colchicine to tubulin; this suggests that the binding site of griseofulvin is distinctly different from that of colchicine. However, we also observed that griseofulvin causes a major change in the conformation of the tubulin molecule, as shown by an increase in the sulfhydryl titer. Interestingly, it does not affect the exposure of the hydrophobic areas of tubulin as determined by binding of the fluorescent probe BisANS. This is an unusual combination of effects because, in general, most ligands that inhibit exposure of hydrophobic areas on tubulin also inhibit exposure of sulfhydryl groups [17, 18]. Therefore, it seems that griseofulvin has a novel mechanism of interaction with tubulin.

MATERIALS AND METHODS

Materials

Griseofulvin and colchicine were purchased from Sigma (St. Louis, MO). BisANS was from Molecular Probes

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† *Abbreviations:* MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonic acid; BisANS, bis-5,5′-[8-(*N*-phenyl)aminonapthalene-1-sulfonic acid]; and FQ, fluorescence quenching.

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(Junction City, OR). All other materials were obtained or purchased as previously described [19]. Griseofulvin was dissolved in DMSO; colchicine and BisANS were dissolved in the buffer immediately prior to experimentation.

Tubulin Preparation

Microtubules were purified from bovine brain cerebra by cycling according to the method of Fellous *et al.* [20]. Tubulin was purified from the microtubules by phosphocellulose chromatography [20]. Unless otherwise indicated, experiments were done in a buffer consisting of 0.1 M Mes, pH. 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM EDTA [20].

Alkylation Reactions

In most experiments, tubulin was reacted with iodo[^{14}C]-acetamide for 2 hr at 37°. After the reaction, tubulin was precipitated by treatment with 5% trichloroacetic acid; the precipitate was collected by filtration and the radioactivity of the filters was determined as previously described [7]. In some experiments, the reaction of tubulin with iodo[^{14}C]acetamide was measured by subjecting the reduced and carboxamidomethylated protein to polyacrylamide gel electrophoresis and then staining with fast green, slicing the gels, and determining the radioactivity of the slices as previously described [21].

Fluorescence

In most experiments, samples of tubulin containing the drugs whose effects were to be tested were incubated at 37°. At certain times, aliquots were withdrawn, made 10 μM in BisANS, and examined in a Hitachi model F-2000 spectrofluorometer. Excitation was at 385 nm; emission was measured at 490 nm. For Scatchard analysis [22], aliquots of tubulin were incubated in the absence or presence of different concentrations of griseofulvin at 37° for 30 min. The samples were placed in the spectrofluorometer and excited at 296 nm. Emission was measured at 336 nm. Griseofulvin does not have any fluorescence at 336 nm, whereas tubulin has a maximum at this wavelength when excited at 296 nm. Fluorescence measurements were corrected for the inner-filter effect according to Lakowicz [23]. The observed fluorescence quenching data were fitted in a nonlinear curve fitting software, MINSQ version 3.2 (Scientific Software, Salt Lake City, UT), using either a one-site or two-site binding equation as follows:

$$F = \sum_{i=1}^{n} (Fmi[D]/[D_{0.5}]_i + [D]),$$

where F is the corrected fluorescence quenching, n is the number of types of ligand binding sites of different affinity on tubulin, Fmi is the maximal fluorescence quenching at site i, [D] is the drug concentration, and $[D_{0.5}]_i$ is the drug concentration at which half-maximal binding occurs at site i. In a single-site model, $[D_{0.5}]$ may be close to the dissociation constant of the drug-tubulin complex if the total drug concentration is much larger than the concentration of bound drug. The assumption is made that all of the tubulin is capable of binding griseofulvin. Just prior to phosphocellulose chromatography, the tubulin is centrifuged at 4°; this allows elimination of very decayed and aggregated tubulin. It is likely, therefore, that all of the tubulin is capable of binding to griseofulvin.

Other Methods

Protein concentrations were determined by a modified form of the procedure of Lowry *et al.* [24], using BSA as a standard [25].

RESULTS

Tryptophan fluorescence quenching was used as a probe to study the interaction of griseofulvin with tubulin. Griseofulvin had a maximum absorbance at 296 nm (Fig. 1A). When the drug and tubulin were excited separately at 296 nm, griseofulvin did not have any fluorescence at 336 nm, whereas tubulin had an emission maximum at 336 nm, which is the wavelength at which tryptophan fluoresces (Fig. 1B). Figure 2 shows that the tryptophan fluorescence was quenched by increasing concentrations of griseofulvin. Upon denaturation of tubulin with 8 M urea, the fluorescence quenching by griseofulvin did not occur (data not shown). The experiment was repeated three times. The results of one of these experiments are shown in Fig. 3. The observed fluorescence quenching data were analyzed by the MINSQ program using one-site and two-site models. Analysis using the two-site model generated one curve almost identical to the curve generated by the one-site model and a second curve that was almost superimposable on the X-axis. The results were thus most consistent with one type of binding site; the one-site model curve that best fits the data is shown in the inset to Fig. 3. Figure 3 shows a Scatchard plot [22] of this experiment from which the K_a could be calculated. Averaging the results of the three experiments, the K_a was $1.26 \pm 0.19 \times 10^4$ M^{-1}. This is based on the assumption that there is not only a single class of griseofulvin binding site but only one such site. It is certainly conceivable that tubulin has two griseofulvin binding sites of equal affinity. In such a case, the calculated free drug concentrations would be slightly different, and thus the Scatchard plots would also differ. We performed this calculation (not shown) and determined that the K_a value would be $1.3 \pm 0.2 \times 10^4$ M^{-1}, statistically indistinguishable from the K_a calculated assuming a single site. In another experiment (not shown), a sample of tubulin was incubated with 100 μM griseofulvin for 30 min; the fluorescence of this sample was measured, and the tubulin was passed through a Sephadex G-25 column to remove the griseofulvin. The fluorescence of the tubulin was measured and

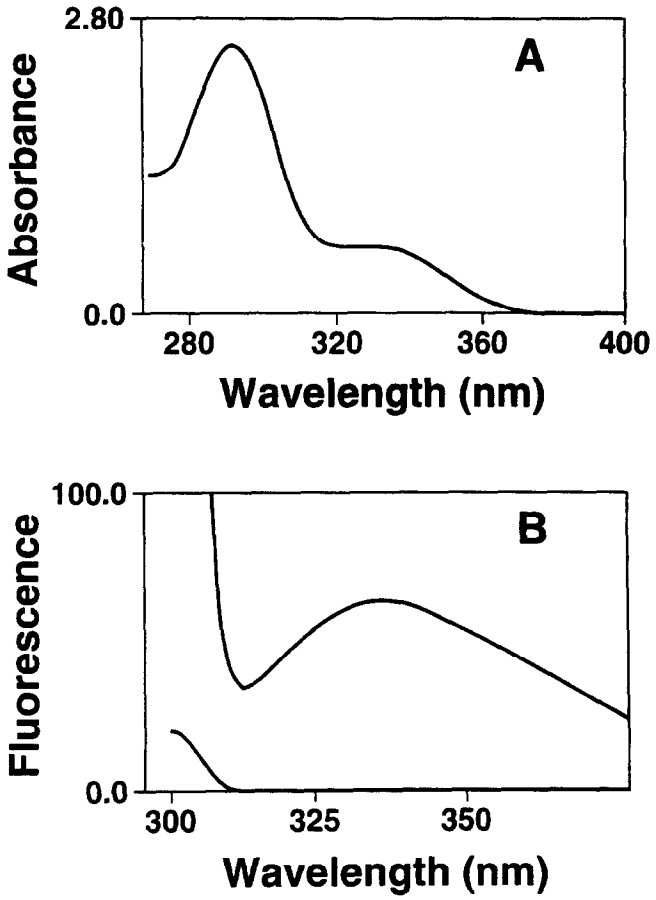

FIG. 1. (A) Excitation spectrum of griseofulvin. Excitation spectrum of 1000 μM griseofulvin in 0.1 M Mes, pH 6.4, 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM EDTA. The maximum absorption was at 296 nm. (B) Emission spectrum of phosphocellulose-purified tubulin and griseofulvin. Upper curve: the emission spectrum of 1 μM phosphocellulose-purified tubulin. Lower curve: the emission spectrum of 100 μM griseofulvin in the same buffer. The excitation wavelength was 296 nm; the emission maximum for tubulin was at 336 nm.

compared with that of an equivalent sample of tubulin that had also been subjected to gel filtration but had not been incubated with griseofulvin. About 90% of the griseofulvin-induced quenching was reversed by gel filtration. By measuring emission at 336 nm, it was possible to show that the concentration of griseofulvin remaining in the sample after gel filtration was 0.0036 μM. This is not enough to account for the 10% quenching that was observed. However, it is clear that griseofulvin binding to tubulin is largely reversible.

Since sulfhydryl groups are sensitive markers for studying the conformation of tubulin and for reporting on the interaction with ligands [17], we measured the effect of griseofulvin on the reaction of tubulin with iodo[^{14}C] acetamide, a reagent that we have shown previously reacts specifically with sulfhydryl groups on tubulin. We found that griseofulvin caused a major enhancement of the exposure of sulfhydryl groups. In the presence of griseofulvin, the

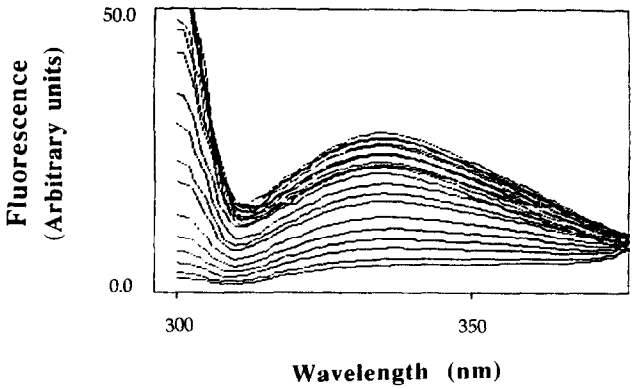

FIG. 2. Fluorescence quenching of tubulin by griseofulvin. Aliquots of phosphocellulose-purified tubulin (1 μM) were incubated with various concentrations of griseofulvin at 37° for 30 min followed by scanning of the emission spectrum. Excitation and emission were at 296 and 336 nm, respectively. The concentrations of griseofulvin (from top to bottom) were as follows (in μM): 0, 0.4, 0.8, 1, 1.5, 2, 3, 4, 8, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 100, and 110.

sulfhydryl titer was twice as much as in the absence of griseofulvin (Table 1). The increase in sulfhydryl titer of tubulin upon interaction with griseofulvin was dependent on drug concentration (Fig. 4). The enhancement affected both the α and β subunit and to about the same extent; the specific radioactivities of both the α and β subunits approximately doubled. The ratio of the specific activity of β to

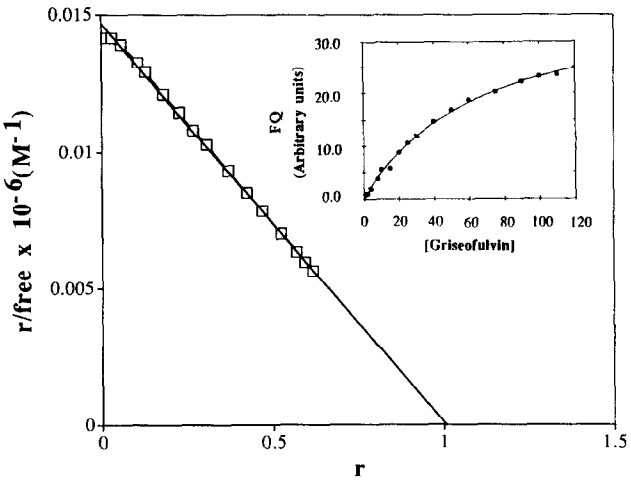

FIG. 3. Scatchard analysis of the binding of griseofulvin to tubulin. Aliquots of phosphocellulose-purified tubulin (1 μM) in buffer were incubated with (0–110 μM) griseofulvin at 37° for 30 min, and the fluorescence quenching of the tubulin was measured at 336 nm. Excitation was at 296 nm. Inset: FQ values at different drug concentrations. The observed fluorescence data were fitted by using a nonlinear curve fitting software, MINSQ, as described under Materials and Methods. The maximum fluorescence quenching of tubulin (1 μM) by griseofulvin was obtained from the curve fitting program. The calculated fluorescence data are presented as a Scatchard plot in which r/free is plotted versus r, where "r" is the amount of griseofulvin bound in mol/mol of tubulin and "free" is the free concentration of griseofulvin at equilibrium.

TABLE 1. Effects of griseofulvin and colchicine on the alkylation of tubulin

Reactions	Iodo [^{14}C]acetamide (mol bound/mol tubulin)
Tubulin + DMSO + iodo[^{14}C]acetamide	2.75 ± 0.10
Tubulin + griseofulvin + iodo[^{14}C]acetamide	5.15 ± 0.07
Tubulin + colchicine + DMSO + iodo[^{14}C]acetamide	1.92 ± 0.14
Tubulin + colchicine + griseofulvin + iodo[^{14}C]acetamide	1.92 ± 0.02

Aliquots (250 μL) of tubulin (0.5 mg/mL) were preincubated for 30 min at 37° in the presence or absence of 250 μM colchicine. After 30 min, each sample was made 500 μM in griseofulvin by addition of 12.5 μL of 10 mM griseofulvin dissolved in DMSO and incubated at 37° for 30 min. To the control sample, 12.5 μL of DMSO was added. After the incubation, 1.83 μL of iodo[^{14}C]acetamide (0.58 Ci/mol) was added to each sample to a final concentration of 500 μM. The incubation at 37° was continued for 2 hr. Incorporation of ^{14}C was determined as described in Materials and Methods. Each combination was done in triplicate. Standard deviations are shown.

TABLE 2. Effect of griseofulvin on the alkylation of the α and β subunits of tubulin

Reactions	Iodo[^{14}C]acetamide (mol bound/mol subunit)	Specific radioactivity of β/α
Tubulin + DMSO + iodo[^{14}C]acetamide	α 2.09 ± 0.23 β 2.19 ± 0.30	0.848 ± 0.042
Tubulin + griseofulvin + iodo[^{14}C]acetamide	α 4.98 ± 0.23 β 4.80 ± 0.12	0.821 ± 0.028

Aliquots (500 μL) of phosphocellulose-purified tubulin (0.5 mg/mL) were incubated at 37° for 30 min with 500 μM griseofulvin by addition of 25 μL of 10 mM griseofulvin dissolved in DMSO. To the control sample, 25 μL of DMSO was added. After 30 min of incubation, 3.66 μL of iodo[^{14}C]acetamide (5.8 Ci/mol) was added to each sample to a final concentration of 500 μM. The incubation at 37° was continued for 2 hr. The samples were dialyzed against buffer, reduced, carboxamidomethylated, and run on a 6% polyacrylamide gel. Gels were stained with fast green. The subunits were scanned at 640 nm to determine the concentrations of the protein. Gels were sliced, and the radioactivity of the slices was determined. Each combination was done in triplicate aliquots. Standard deviations are shown.

that of α was not affected significantly by the presence of griseofulvin (Table 2). We have observed previously that the sulfhydryl titer of tubulin varies from one preparation to another. Comparison of Tables 1 and 2 shows that this is true. The important point, however, is that in each batch of tubulin griseofulvin caused a major increase in sulfhydryl titer.

Since griseofulvin had effects on tubulin so strikingly different from those of other known tubulin ligands such as colchicine, it was of interest to examine the effect of colchicine in combination with griseofulvin. The enhancing effect of griseofulvin on sulfhydryl titer exposure was strongly countered by colchicine. There was no change in the sulfhydryl titer of the tubulin–colchicine complex in the presence of griseofulvin (Table 1). However, griseofulvin was able to quench the tryptophan fluorescence of the colchicine–tubulin complex (Fig. 5).

We have noted previously a strong correlation between the effect of a ligand on the alkylation of tubulin sulfhydryls and its effect on the exposure of hydrophobic areas on tubulin, which can be estimated by interaction with the fluorescent probe BisANS. If griseofulvin enhances exposure of tubulin sulfhydryl groups, one would expect that it

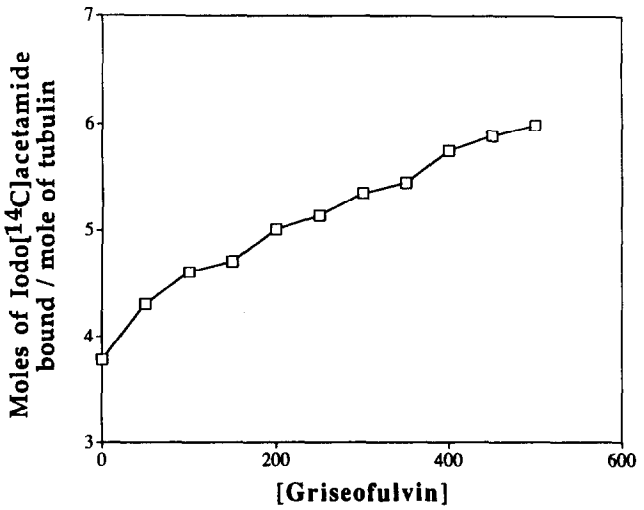

FIG. 4. Effect of different concentrations of griseofulvin on the alkylation of tubulin. Aliquots (250 μL) of tubulin (0.5 mg/mL) were incubated in the absence or presence of different concentrations of griseofulvin (0–500 μM) at 37° for 30 min. The volume of griseofulvin was 12.5 μL. As a control, instead of griseofulvin, 12.5 μL of DMSO was added. After the incubation, 1.83 μL of iodo[^{14}C]acetamide (0.58 Ci/mol) was added to each sample to a final concentration of 500 μM. The incubation at 37° was continued for 2 hr. Incorporation of ^{14}C was determined as described in Materials and Methods. Each combination was done in triplicate.

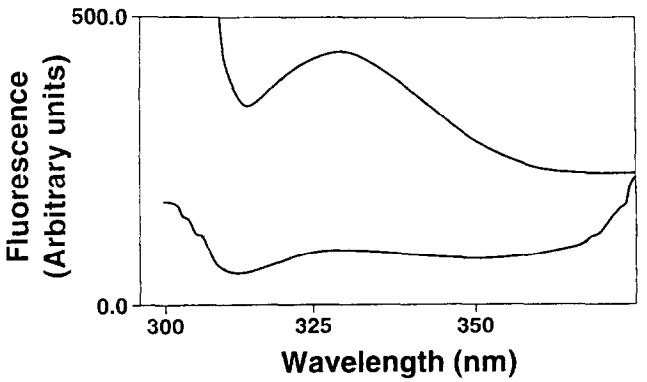

FIG. 5. Emission spectrum of the tubulin–colchicine complex in the presence or absence of griseofulvin. Aliquots of 1 μM tubulin in buffer were incubated with 50 μM colchicine at 37° for 30 min followed by incubation in the absence or presence of 100 μM griseofulvin at 37° for 30 min. Fluorescence was measured at 336 nm. Excitation was at 296 nm. The upper curve indicates the tubulin–colchicine complex in the absence of griseofulvin; the lower curve shows the tubulin–colchicine complex in the presence of griseofulvin.

would also enhance exposure of hydrophobic areas. Figure 6 shows that this is not the case. Griseofulvin by itself had no effect on the binding of BisANS to tubulin. In the experiment shown in Fig. 6, the concentration of griseofulvin was 500 μM. However, a range of griseofulvin concentrations of 20–200 μM did not have any effect on BisANS binding (not shown). In contrast, in the presence of colchicine, griseofulvin caused a small decrease in the binding of BisANS (Fig. 6).

DISCUSSION

The effects of griseofulvin on tubulin, as reported in the literature, are contradictory. In 1973, Grisham *et al.* [9] reported that griseofulvin does not cause disruption of microtubules, though Wilson and Bryan in the same year [26] found that griseofulvin inhibits assembly of microtubules in the absence of microtubule-associated proteins. There are reports in the literature suggesting that griseofulvin binds either to tubulin itself [11, 12] or to one or more of the microtubule-associated proteins [13] that are known to associate with microtubules both *in vivo* [14] and *in vitro* [15].

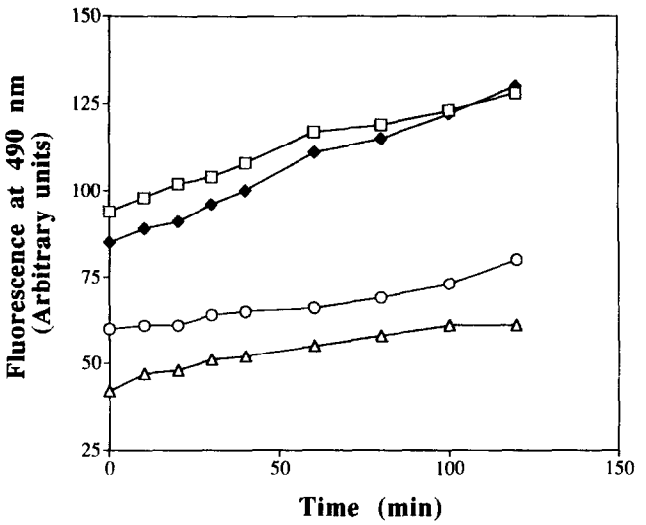

FIG. 6. Effects of griseofulvin and colchicine on BisANS binding to tubulin. Two samples (9 mL) of tubulin (0.2 mg/mL) were preincubated at 37° for 30 min in the absence or presence of 500 μM griseofulvin. Another two samples (9 mL) of tubulin (0.2 mg/mL) were also preincubated at 37° for 30 min in the absence or presence of colchicine (100 μM) followed by incubation with or without 500 μM griseofulvin at the same temperature for 30 min. After the preincubations were over, incubation continued at 37°. At the indicated times, 1-mL aliquots were withdrawn and mixed with 5 μL BisANS (to give a final concentration of 10 μM). The aliquots were placed in a fluorescence cuvette, and fluorescence intensity was measured. Excitation and emission were at 385 and 490 nm, respectively. The emission values were corrected for the sample volume and for the fluorescence that would have been obtained if tubulin had not been present. Key: (□) tubulin + DMSO, (◆) tubulin + griseofulvin, (○) tubulin + colchicine + DMSO, and (△) tubulin + colchicine + griseofulvin.

In the present study, we have examined the interaction of griseofulvin with tubulin. To study the interaction fluorometrically, we had first to determine the wavelength where griseofulvin gives maximal absorbance. Fig. 1A shows that griseofulvin absorbed maximally at 296 nm. The emission spectrum of tubulin had a maximum at 336 nm, corresponding to tryptophan fluorescence; in contrast, griseofulvin did not emit at this wavelength (Fig. 1B). When tubulin was treated with different concentrations of griseofulvin at 37° for 30 min, we found that the tryptophan fluorescence of tubulin was decreased with increasing concentrations of the drug (Fig. 2). This suggests that there is a gross conformational change as a result of binding of griseofulvin to tubulin or that a tryptophan residue is in or very near the binding site. Denaturation of tubulin with urea abolished the quenching caused by griseofulvin, suggesting that tryptophan fluorescence quenching by griseofulvin requires that tubulin be in its native conformation. From the quenching values, the affinity constant (K_a) was determined by Scatchard analysis. The affinity constant was $1.26 \pm 0.19 \times 10^4\ M^{-1}$, and the binding was monophasic in nature consistent with only a single class of binding site for griseofulvin on the tubulin molecule. The affinity of griseofulvin for tubulin appeared to be low compared with those of colchicine ($K_a = 1.1 \times 10^7$) [27] and podophyllotoxin ($K_a = 1.8 \times 10^6$) [28]. Tubulin–griseofulvin binding also appeared to be largely reversible. The 10% that was not reversible may reflect tubulin molecules aggregating and trapping the griseofulvin molecules within the aggregate. Also, it is conceivable that the incubation with griseofulvin could have accelerated decay in the tubulin sample beyond that of the control sample. In another study, we have shown that the drug IKP104, which greatly enhances alkylation, also accelerates decay [29].

Griseofulvin has a weaker affinity for tubulin than do other anti-tubulin drugs such as colchicine and vinblastine. Perhaps this weakness accounts for some of the contradictions in the literature about griseofulvin. It is likely that griseofulvin resembles other drugs in that it blocks mitosis by stabilizing microtubule dynamics at concentrations far below those at which it induces microtubule depolymerization. Thus, in the study of Grisham *et al.* [9], griseofulvin blocked mitosis without depolymerizing microtubules, while under simpler *in vitro* conditions griseofulvin inhibited microtubule assembly [26].

Griseofulvin appears to cause a major change in the conformation of tubulin as measured by the exposure of sulfhydryl groups (Table 1); the magnitude of this enhancement is on the order of 100%. This increment of sulfhydryl titer is dependent on the griseofulvin concentration (Fig. 4). When we studied the effect of griseofulvin on the alkylation of the α and β subunits of tubulin (Table 2), we found that griseofulvin affected both of the subunits of tubulin equally. Ligand-induced enhancement of tubulin alkylation has been observed before, with single-ring analogues of colchicine, such as 3,4,5-trimethoxybenzaldehyde; in the latter case, however, 100% enhancement required

ligand concentrations of over 2 mM [30]. In general, tubulin ligands do not enhance alkylation [7, 31]. The striking exception is IKP104, which has an effect similar to that of griseofulvin, but is structurally very dissimilar [29] and, as will be seen below, differs from griseofulvin in its effect on the hydrophobic areas of tubulin.

Since griseofulvin interacts differently with tubulin than do many other drugs, we decided to study the effect of another drug, namely colchicine, in combination with griseofulvin. Table 1 shows that there was no change in the sulfhydryl titer of the tubulin–colchicine complex in the presence of griseofulvin. Figure 5 shows that the griseofulvin binding site on tubulin is distinctly different from the colchicine site since griseofulvin quenched the tryptophan fluorescence of the colchicine–tubulin complex; this supports the earlier observation of Wilson [32], who also found that griseofulvin does not prevent the binding of colchicine to tubulin. Thus, although griseofulvin bound to the tubulin–colchicine complex, it did not alter the conformation of at least that portion of the tubulin molecule where the extra sulfhydryls were exposed. It may be that the mechanism of interaction of griseofulvin with tubulin is distinctly different from its interaction with the tubulin–colchicine complex; this is consistent with the fact that colchicine changes the conformation of tubulin [27].

Usually, there is a striking correlation between the effect of a ligand on the alkylation of sulfhydryl groups of tubulin and its effect on the exposure of hydrophobic areas on tubulin. Drugs such as podophyllotoxin, colchicine, and vinblastine, which inhibit alkylation, also inhibit exposure of hydrophobic areas; in both systems, the effect of vinblastine is larger than that of colchicine and the effect of colchicine is larger than that of podophyllotoxin [31, 33]. Along the same lines, maytansine and spongistatin 3, which have no effect on alkylation, also have no effect on exposure of hydrophobic areas [7, 33, 34]. IKP104 strongly enhances both effects [29]. The only exceptions to this correlation that we have observed are halichondrin B, which enhances exposure of hydrophobic areas (by up to 64%) without affecting the sulfhydryl groups, and homohalichondrin B, which inhibits alkylation by 11% without affecting exposure of hydrophobic areas [35]. Griseofulvin clearly constitutes another exception in that it enhances alkylation without affecting hydrophobic areas. However, this particular pattern has not been observed with other tubulin ligands; a further complexity is that it does not affect the alkylation of the tubulin–colchicine complex. Thus, it appears that griseofulvin has a unique combination of effects on the tubulin molecule and would therefore be a useful probe for examining the conformational properties of tubulin.

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