Interaction of Bovine Brain Tubulin with the 4(1*H*)-Pyrizinone Derivative IKP104, an Antimitotic Drug with a Complex Set of Effects on the Conformational Stability of the Tubulin Molecule†

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ABSTRACT: The ligands of tubulin have proved to be excellent probes for the conformation of the tubulin molecule. The most varied in their effects on tubulin are those ligands which are competitive or noncompetitive inhibitors of vinblastine binding. The 4(*H*)-pyrizinone derivative 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone (IKP104) is a novel antimitotic drug which inhibits microtubule assembly *in vitro* and *in vivo* and polymerizes tubulin into spiral filaments. Using a fluorescence assay, we found that IKP104 appears to bind to tubulin at two classes of site, differing in affinity. IKP104 also blocks formation of an intrachain cross-link in β-tubulin, induced by *N,N'*-ethylenebis(iodoacetamide), linking Cys^{12} to either Cys^{201} or Cys^{211}. IKP104 appears to belong to that group of tubulin ligands which includes vinblastine, maytansine, rhizoxin, phomopsin A, dolastatin 10, and halichondrin B. An unusual effect of IKP104 is that it greatly enhances the decay or apparent unfolding or opening of the tubulin molecule. The sulfhydryl titer of tubulin is doubled and the exposure of hydrophobic areas on the tubulin molecule is tripled by IKP104. These effects of IKP104 are counteracted by vinblastine, maytansine, and phomopsin A, suggesting that IKP104 may be competing with these other drugs for binding to tubulin. However, the effects are also counteracted by colchicine and podophyllotoxin, implying a more complex effect, namely, that IKP104 and colchicine, even when both are bound to tubulin, are competing for their effects on the same domain of tubulin. Surprisingly, when IKP104 is used in conjunction with colchicine, binding of colchicine to tubulin is strongly stabilized. It appears that IKP104 has an unusual and complex effect on the conformation of the tubulin molecule, perhaps stabilizing the domain to which it binds and simultaneously destabilizing the domain to which colchicine binds.

Tubulin, the subunit protein of microtubules, binds to a number of antimitotic drugs (Dustin, 1984). Besides their common ability to inhibit microtubule assembly, these drugs have a variety of other effects on the tubulin molecule. The sulfhydryl groups of tubulin are very sensitive and discriminating markers for the interactions of ligands with tubulin (Luduena & Roach, 1991). Tubulin reacts with the bifunctional molecule *N,N'*-ethylenebis(iodoacetamide) (EBI)[1] to form two intrachain cross-links in β-tubulin (Luduena & Roach, 1981a; Roach & Luduena, 1984). These cross-links create tubulin species of discrete electrophoretic mobilities, which allows for ready monitoring of the cross-linking reaction. One of these cross-links, designated β*, is between Cys^{239} and Cys^{354}; the second cross-link, designated β^s, is between Cys^{12} and either Cys^{201} or Cys^{211} (Little & Luduena, 1985, 1987). Different ligands affect formation of the cross-links differently. In addition to the cross-links, tubulin sulfhydryl groups become exposed and reactive with iodo-[^{14}C]acetamide as tubulin decays; ligands have discrete effects on decay (Luduena & Roach, 1981a,b). Decay is also reflected in the increase in exposure of hydrophobic areas on the surface of tubulin; this can be monitored using the fluorescent probe BisANS.

Tubulin ligands fall into three broad categories, as follows: (1) colchicine, podophyllotoxin, and related drugs inhibit β* formation, enhance β^s formation, and inhibit both BisANS binding and alkylation with iodo[^{14}C]acetamide; (2) GTP, vinblastine, maytansine, and ligands that are competitive or noncompetitive inhibitors of vinblastine inhibit β^s formation, enhance β* formation, and have mixed effects on alkylation and BisANS binding; and (3) ligands that bind elsewhere (such as BisANS at its high-affinity site) have little effect on cross-link formation or alkylation (Luduena & Roach, 1981a; Roach & Luduena, 1984; Luduena et al., 1986a; Prasad et al., 1986). Within the second category, there are various subcategories, as follows: (a) vinblastine inhibits β^s formation only partially and strongly inhibits alkylation and BisANS binding; (b) maytansine and rhizoxin, presumably competitive inhibitors of vinblastine binding, completely inhibit β^s formation and have no effect on

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[1] Abbreviations: BisANS, bis-5,5'-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid]; EBI, *N,N'*-ethylenebis(iodoacetamide); IKP104, 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone.

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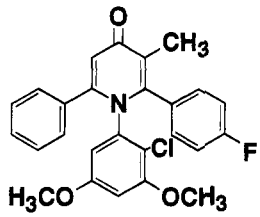

FIGURE 1: Structure of IKP104.

alkylation or BisANS binding; (c) phomopsin A and dolastatin 10, noncompetitive inhibitors of vinblastine binding, completely inhibit β^s formation and inhibit alkylation and BisANS binding even more strongly than does vinblastine; (d) halichondrin B, a noncompetitive inhibitor of vinblastine binding, strongly inhibits β^s formation, has no effect on alkylation, and enhances BisANS binding by 64%; and (e) homohalichondrin B, a close structural analogue to halichondrin B, completely inhibits β^s formation, has little effect on BisANS binding, and weakly inhibits alkylation (Luduena & Roach, 1981c; Roach & Luduena, 1984; Prasad et al., 1986; Sullivan et al., 1990; Luduena et al., 1989, 1990, 1992, 1993).

A recently developed antitumor drug is the 4(1*H*)-pyrizinone derivative 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone (IKP104) (Figure 1) (Mizuhashi et al., 1990). When B16 melanoma cells were treated with 8 μM IKP104, normal mitosis was inhibited and mitotic cells exhibited scattered chromosomes with no mitotic spindles; electron microscopic examination showed that the mitotic and interphase microtubules disappeared, in some cases being replaced by bundles of protofilaments (Mizuhashi et al., 1991). IKP104 was also found to inhibit microtubule assembly *in vitro* with an IC_{50} of 1.31 μM (Mizuhashi et al., 1992). Addition of IKP104 to microtubules *in vitro* caused them to disassemble into spiral polymers, similar to those seen when vinblastine is added to preparations of microtubule protein. Analysis of peptide maps obtained by digesting tubulin in the presence of various ligands showed that the pattern seen with IKP104 was very similar to that obtained in the presence of vinblastine but distinctly different from that generated in the presence of colchicine. It was proposed, therefore, that IKP104 may bind to tubulin at or near the vinblastine-binding site (Mizuhashi et al., 1992).

We have examined the interaction of IKP104 with tubulin using a variety of approaches. We found that IKP104 bound to tubulin with kinetics consistent with two classes of site of different affinity. IKP104 also inhibited vinblastine-induced tubulin stabilization and formation of the EBI-induced β^s cross-link. For these reasons, IKP104 appears to belong to the same category of tubulin ligands as vinblastine, maytansine, phomopsin A, dolastatin 10, halichondrin B, and homohalichondrin B. However, we also observed IKP104 caused a major enhancement of tubulin decay, as measured by increased sulfhydryl titer and BisANS binding and by rapid loss of the ability to bind to [^{3}H]-colchicine. The enhancement was as much as 3-fold. In fact, IKP104 appeared to complete the conformational change which is involved in tubulin decay. No other drug is known to cause such a large increase in tubulin decay.

The most unexpected effect of IKP104, however, was that whereas tubulin incubated with IKP104 rapidly lost its ability to bind [^{3}H]colchicine, when tubulin was coincubated with both IKP104 and colchicine, the ability of tubulin to bind to colchicine increased dramatically. In short, although IKP104 destabilized tubulin when used by itself, it strongly stabilized tubulin when used in conjunction with colchicine. It seems, therefore, that IKP104 has a unique mechanism of interaction with tubulin. IKP104 may be a useful probe for examining the conformational states which are available to the tubulin molecule.

These results have been presented in preliminary form elsewhere (Prasad et al., 1993).

MATERIALS AND METHODS

Materials. IKP104 was from the K-I Research Institute (Shizuoka, Japan). Vinblastine was a kind gift from the Eli Lilly Corporation (Indianapolis, IN). Maytansine was obtained from Dr. Matthew Suffness of the Drug Development Program, National Cancer Institute. Phomopsin A, isolated as previously described (Culvenor et al., 1977), was purchased from Dr. J. A. Edgar. [^{3}H]Colchicine was from New England Nuclear (Boston, MA). BisANS was from Molecular Probes (Junction City, OR). All other materials were obtained or purchased as previously described (Luduena et al., 1982). IKP104, podophyllotoxin, phomopsin A, and maytansine were dissoved in dimethyl sulfoxide immediately prior to experimentation.

Tubulin Preparation. Microtubules were purified from bovine brain cerebra by cycling according to the method of Fellous et al. (1977). Tubulin was purified from the microtubules by phosphocellulose chromatography. Unless otherwise indicated, experiments were done in a buffer consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (Fellous et al., 1977).

Cross-Linking. Tubulin was treated with EBI and then dialyzed, reduced, carboxymethylated, and subjected to polyacrylamide gel electrophoresis. The system of Laemmli (1970) was used for determining the yield of the β^* cross-link, while the modified verison of Banerjee et al. (1987) was used to measure the yield of the β^s cross-link. These yields were calculated as previously described (Roach & Luduena, 1984). Tubulin samples contained reduced and carboxymethylated conalbumin as an internal standard (Luduena & Roach, 1981a). On both of these gel systems, β-tubulin migrates as two closely spaced bands, designated as β_1 and β_2. β_1, the band which migrates faster, consists of the β_I, β_{II}, and β_{IV} isotypes; the β_2 band, which migrates just behind β_1, consists of the β_{III} isotype (Banerjee et al., 1988). β_1 is the source of the β^* and β^s bands; the β_2 band does not participate in the formation of these cross-links (Luduena et al., 1982; Roach & Luduena, 1984; Sharma & Luduena, 1994). In some experiments, the yield of the residual non-cross-linked β_1 band was measured and also the yield of high-molecular-weight cross-linked aggregated tubulin. This latter species arises from nonspecific cross-linking and does not migrate onto a gel. Its yield is measured indirectly, using the conalbumin internal standard, as previously described (Roach & Luduena, 1984).

Alkylation. Tubulin was reacted with iodo[^{14}C]acetamide for 1 h at 37 °C. 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 (Luduena & Roach, 1981c).

Fluorescence Measurements. Samples of tubulin containing the drugs whose effect was to be tested were incubated at 37 °C. At certain times, aliquots were taken, made 10 μM in BisANS, and examined in a SPF500C spectrofluorometer (SLM) set in the ratio mode; excitation was at 385 nm and emission was measured at 490 nm (Prasad et al., 1986). For measurement of the binding of IKP104 to tubulin, aliquots of tubulin (2 μM) were incubated in the presence of different concentrations of IKP104 at 37 °C for 30 min. The samples were placed in a cuvette in a Hitachi model F-2000 spectrofluorometer and excited at 278 nm. IKP104 had a maximum fluorescence at 467 nm. In the presence of tubulin, the fluorescence peak of IKP104 shifts toward blue (from 467 to 451 nm) with the increment of fluorescence intensity also shifting. The difference in fluorescence intensity between tubulin−IKP104 and IKP104 at a particular concentration at 451 nm was measured and corrected for the inner-filter effect according to Lakowicz (1983). The corrected observed fluorescence data were analyzed using either a one-, two-, or three-site binding equation as follows:

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

where F is the observed corrected fluorescence value at any drug concentration [D], n is the assumed number of classes of IKP104 binding sites of different affinity on tubulin, F_{mi} is the fluorescence value at site i at the saturating IKP104 concentration, and $[D_{0.5}]_i$ is the drug concentration at which half of the tubulin molecules are bound to the drug at site i. In a single-site model $[D_{0.5}]$ could approximate the dissociation constant for the IKP104−tubulin complex. For a two- or three-site model, the interpretation is more complex, and $[D_{0.5}]$ should at best be considered an apparent K_d. The data were fitted to each of the one-, two-, and three-site models using the nonlinear curve-fitting software MINSQ, version 3.2 (Micromath Scientific Software, Salt Lake City, UT).

Other Methods. The binding of [^{3}H]colchicine to tubulin was determined by filtration using DEAE-cellulose filters (Borisy, 1972). Binding of [^{3}H]vinblastine was measured by the nonequilibrium method of Wilson et al. (1975). Protein concentrations were determined by a modified form of the procedure of Lowry et al. (1951) using bovine serum albumin as a standard (Schacterle & Pollack, 1973).

RESULTS

Measurement of the Binding of IKP104 to Tubulin. We determined the absorption spectrum of IKP104 and found that it had two absorption maxima, at 236 and 278 nm. When IKP104 was excited at 278 nm, maximum emission was at 467 nm. However, when IKP104 was bound to tubulin, the emission maximum shifted toward 451 nm. Denaturation of tubulin with 8 M urea abolished this shift. In order to estimate the binding, we incubated tubulin (2 μM) with a series of concentrations of IKP104 (0−20 μM). The experiment was repeated three times. The results of one of these experiments are shown in Figure 2; the observed fluorescence values were corrected for the inner-filter effect (Lakowicz, 1983). The data were analyzed by the MINSQ program using one-, two-, and three-site models, making the simplifying assumption that the sites do not influence each other. In each of the three experiments, the spread of the residuals was much smaller in the two-site model than in the one-site model. Analysis using the three-site model generated two curves almost identical to the two generated by the two-site model and a third curve that was vanishingly small, almost superimposable on the x-axis. The results were thus most consistent with two types of binding site; the two-site model curve that best fits the data is shown in Figure 2 together with its two component curves from which the following parameters could be readily derived and averaged for the three experiments. The values of $[D_{0.5}]$ for the high- and low-affinity binding sites were, respectively, 0.21 ± 0.09 μM and 17 ± 4 μM. Interestingly, the low-affinity binding site gave the greatest fluorescence. The F_m values (in arbitrary units) for the high- and low-affinity sites were, respectively, 4.4 ± 0.6 and 36 ± 6. The stoichiometries of binding at the two classes of site were not ascertained.

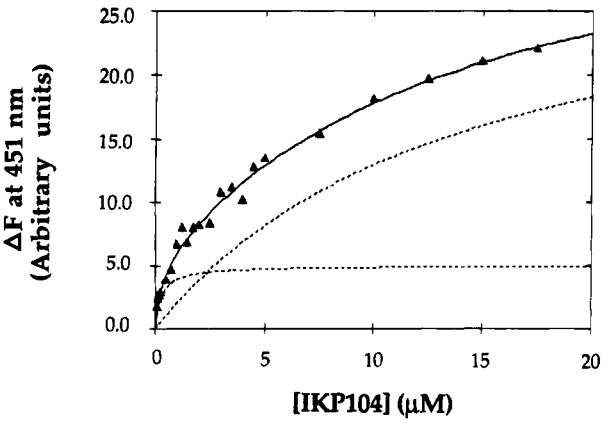

FIGURE 2: Analysis of the binding of IKP104 to tubulin. Aliquots of tubulin (2 μM) were incubated with a series of concentrations of IKP104 (10−20 μM) at 37 °C for 30 minutes and then excited at 278 nm. Emission at 451 nm was measured. The emission at 451 nm of the same series of IKP104 concentrations, in the absence of tubulin, was also measured. The values were corrected for the inner filter effect (Lakowicz, 1983). The corrected fluorescence data were fitted to a two-site model by using the nonlinear curve-fitting software MINSQ as described under Materials and Methods. The hypothetical curves for each type of binding site are shown as dashed lines.

Effect of IKP104 on the Intrachain Cross-Linking of β-Tubulin. In order to identify the family of tubulin ligands to which IKP104 belongs, we examined its effect on the EBI-induced formation of the β^s and β^* cross-links. We found that IKP104 inhibited formation of both cross-links by EBI (Figure 3). This was apparently a unique effect since no other ligand inhibits formation of both cross-links. However, a close look at the results of the cross-linking experiment suggested that IKP104's inhibition of β^s formation was a direct and specific effect, while its inhibition of β^* formation was indirect and nonspecific. One would expect that if a ligand specifically inhibits transformation of the β_1 band into a faster-moving cross-linked product, then the decreased yield of the product would be matched by a corresponding increased recovery of the residual or non-cross-linked β_1 band. If the yields of both the product and the reactant are lower, then one might expect that the ligand induces formation of a different product and that its apparent inhibition of formation of the cross-linked product of interest would be a secondary consequence of enhancing the forma-

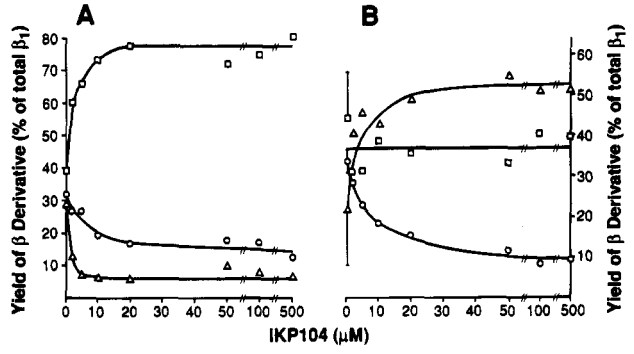

FIGURE 3: Effect of IKP104 concentration on the EBI-induced cross-linking of tubulin. Aliquots (250 μL) of tubulin (0.66 mg/mL), purified in the absence of GTP and $MgCl_2$, containing reduced and carboxymethylated conalbumin (0.20 mg/mL) were incubated with 0.91 mM EBI in the presence of either 1 mM GTP (A) or 50 μM podophyllotoxin (B). Samples were processed as described in Materials and Methods. Panel A shows the yield of the β* cross-link (○), the non-cross-linked β_1 (△), and the aggregated cross-linked β_1 (□). Panel B shows the yield of the β^s cross-link (○), the non-cross-linked β_1 (△), and the aggregated cross-linked β_1 (□). Samples with 0 μM IKP104 were done in duplicate; the average of the two values is shown, together with the range.

tion of a different product. Analysis of the experiment in Figure 3 shows that EBI induced formation of a great deal of high molecular weight cross-linked aggregate in the presence of IKP104. As seen in Figure 3A, the inhibition of β* formation was not accompanied by a concomitant increase in non-cross-linked β_1 but rather by a great decrease in non-cross-linked β_1 and a very great increase in aggregated cross-linked β_1. In contrast, IKP104's inhibition of β^s formation was a specific effect (Figure 3B); a decrease in the yield of β^s was accompanied by an increase in the yield of non-cross-linked β_1 and relatively little change in aggregated cross-linked β_1.

In the experiment shown in Figure 3, the tubulin was purified in the absence of GTP or $MgCl_2$ in order to permit measurement of the yield of β^s (which does not form in the presence of GTP). One could argue that the resulting greater instability of the tubulin could mask a specific inhibition of β* formation. To deal with this possibility, tubulin was purified in the presence of GTP and $MgCl_2$ and the effect of IKP104 on β* formation was determined. The results (not shown) indicated that as the IKP104 concentration increased from 0 to 100 μM, the yield of β* fell from 76% to 51%; however, there was no corresponding increase in non-cross-linked β_1, which in fact fell from 21% to 5%. The yield of aggregated cross-linked β_1 rose from 3% in the absence of IKP104 to 45% in the presence of 100 μM IKP104.

Effect of IKP104 on the Alkylation of Tubulin Sulfhydryls by Iodo[^{14}C]acetamide. The results of the cross-linking experiments suggested that IKP104 caused increased exposure of those sulfhydryl groups not involved in the B^s cross-link. In order to examine that directly, we measured the effect of IKP104 on the reaction of tubulin with iodo[^{14}C]-acetamide, a reagent which we have previously shown reacts specifically with sulfhydryl groups in tubulin. We found that IKP104 caused a major enhancement of the rate of exposure of sulfhydryl groups. In the presence of IKP104 the sulfhydryl titer could be twice as much as in the absence of IKP104 (Figure 4). Figure 4 also shows that in the presence of IKP104, the sulfhydryl titer reached a maximum level at about 10 h of incubation. When the effect of

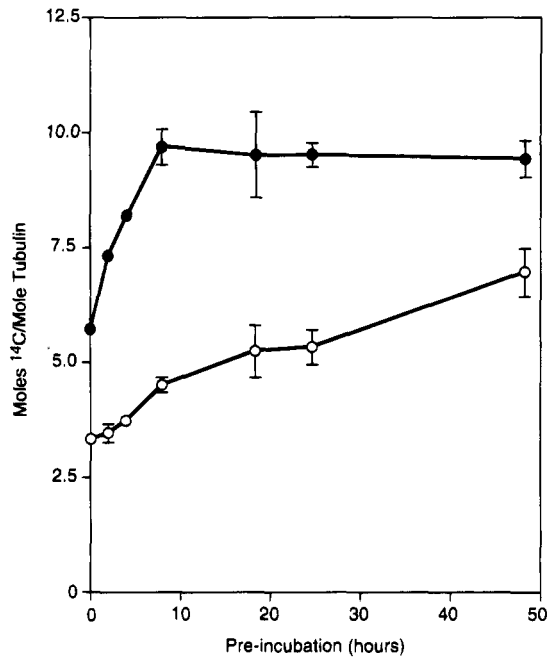

FIGURE 4: Effect of IKP104 on the rate of exposure of tubulin sulfhydryl groups. Samples (7.0 mL) of tubulin (0.825 mg/mL) were incubated at 25 °C in the presence (●) or absence (○) of 10 μM IKP104. At the indicated times, aliquots (200 μL) were removed. Iodo[^{14}C]acetamide (0.467 Ci/mol) was added to each aliquot to give final concentrations of tubulin and iodo[^{14}C]-acetamide of 0.66 mg/mL and 1.36 mM, respectively. Incubation was continued for 1 h at 37 °C. At the end of the hour, samples were precipitated with 5% trichloroacetic acid and filtered. The radioactivity of the filters was determined. Aliquots were taken in quadruplicate. Standard deviations are shown.

sulfhydryl titer was examined as a function of the IKP104 concentration (Figure 5A–E), it appeared that, in 1 h at 37 °C, half-maximal exposure of sulfhydryls was obtained at an IKP104 concentration of 2.2 ± 0.4 μM. Since IKP104 had effects on tubulin so strikingly different from those of other known tubulin ligands, it was of interest to compare the effects of these ligands in combination with IKP104. The enhancing effect of IKP104 on sulfhydryl titer exposure was strongly countered by colchicine, podophyllotoxin, and phomopsin A (Figures 5A–C). Even raising the concentration of IKP104 to 100 μM did not abolish the effects of these drugs. In contrast, with vinblastine and maytansine, there appeared to be some competition. As shown in Figure 5D, the inhibitory effects of 10 μM vinblastine were evident at low concentrations of IKP104 but were completely abolished at 20 μM IKP104. Competition by maytansine was not so apparent (Figure 5E); although maytansine by itself had no effect on tubulin alkylation, as we have previously reported (Luduena & Roach, 1981c), its presence was clearly sufficient to negate the enhancing effect of IKP104, and in fact, maytansine was more effective than vinblastine. As seen in Figure 5E, 20 μM IKP104 caused a very slight enhancement of alkylation in the presence of 10 μM maytansine. Furthermore, 100 μM IKP104 caused 102% enhancement of alkylation in the absence and 52% enhancement in the presence of 10 μM maytansine. In other words, 100 μM IKP104 could significantly but not completely overcome the effect of 10 μM maytansine.

Effect of IKP104 on the Exposure of Hydrophobic Areas on the Tubulin Molecule. We have previously noted an almost perfect correlation between the effect of a ligand on

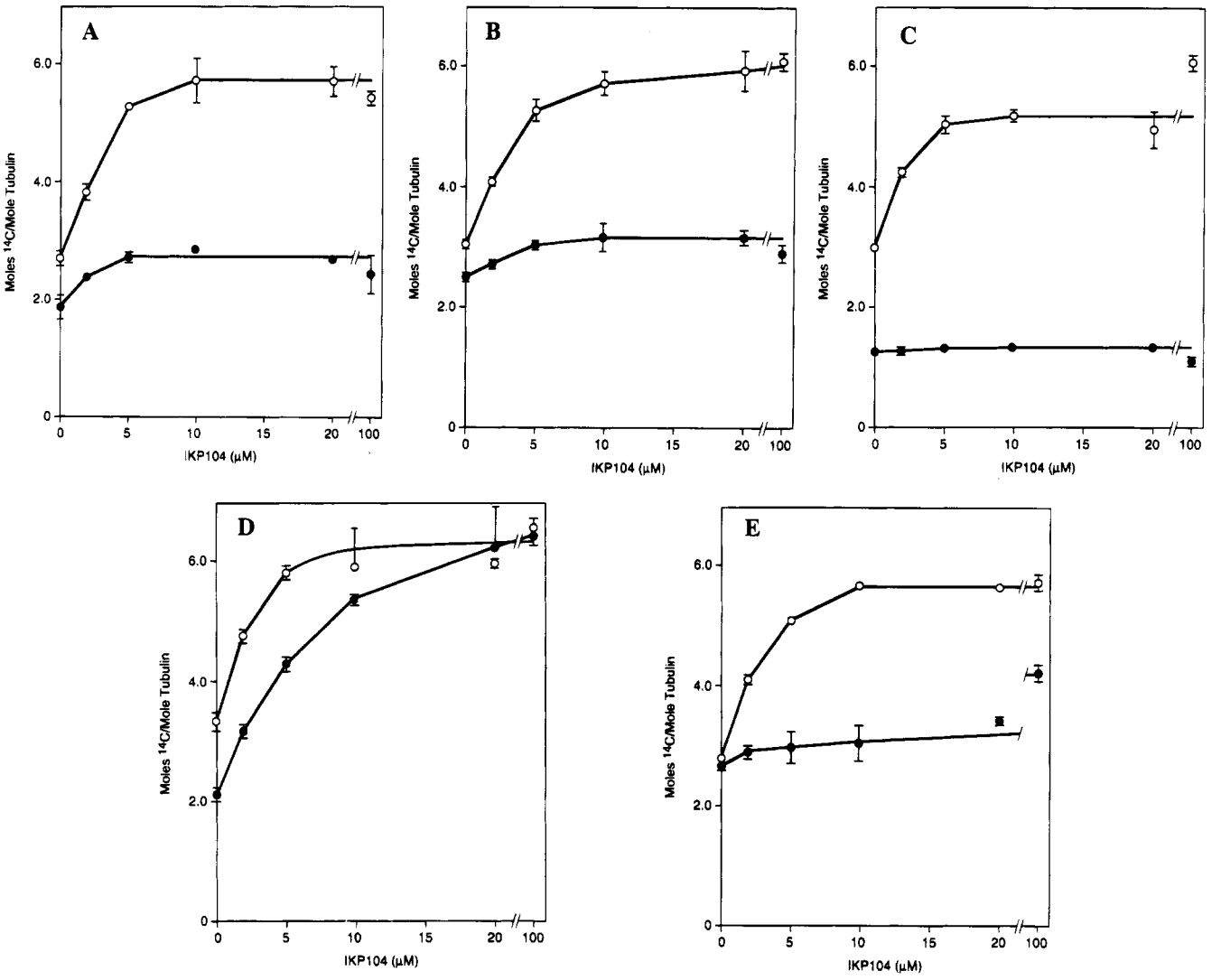

FIGURE 5: Effect of our ligands on the interaction of IKP104 with tubulin. Aliquots (250 μL) of tubulin (0.66 mg/mL) were incubated for 1 h at 37 °C with the indicated concentrations of IKP104 and in the presence of 1.36 mM iodo[^{14}C]acetamide (0.46 Ci/mol). Incubations were in the presence (●) or absence (○) of 10 μM concentrations of either colchicine (A), podophyllotoxin (B), phomopsin A (C), vinblastine (D), or maytansine (E). The values shown in the presence of 100 μM IKP104 were obtained in a separate experiment and normalized to the other results in each figure by multiplying the measured values by the ratio of the control (no IKP104, no ligand) value shown in the figure to that of the experiment using 10 μM IKP104. In this latter experiment, the control value was 3.09 ± 0.08 mol of ^{14}C/mol of tubulin; the value obtained in the presence of 100 μM IKP104 alone was 6.26 ± 0.14 mol of ^{14}C/mol of tubulin. The values obtained in the presence of 100 μM IKP104 and 10 μM concentrations of the other ligands were as follows (in moles of ^{14}C/mole of tubulin): colchicine, 2.82 ± 0.39; podophyllotoxin, 2.94 ± 0.15; phomopsin A, 1.12 ± 0.06; vinblastine, 6.11 ± 0.14; maytansine, 4.70 ± 0.16. All incubations were done in triplicate, except for the one with 10 μM maytansine and 20 μM IKP104 in panel E, which was done in duplicate. Standard deviations are shown.

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 IKP104 enhances exposure of tubulin sulfhydryl groups, one would predict that it would also enhance exposure of hydrophobic areas. Figures 6–8 show that this was indeed the case. In the presence of IKP104, the amount of BisANS binding was enhanced by as much as 3-fold. In many experiments, the level of exposure of hydrophobic areas appeared to have reached a maximum.

When the effects of ligand combinations were tested in this system, their effects paralleled those on the sulfhydryl groups. The suppression caused by 10 μM phomopsin A appeared to be unaffected by 10 μM IKP104 (Figure 6); even the initial decrease of BisANS binding induced by phomopsin A was unaffected. In contrast, the effect of 10 μM vinblastine was abolished by 10 μM IKP104 (Figure 6). Identical results were obtained in an experiment using 20 μM concentrations of phomopsin A, vinblastine, and IKP104 (not shown). The effects of IKP104 and vinblastine appeared to compete. In other words, at a given IKP104 concentration, increasing the vinblastine concentration decreased the enhancement of fluorescence. For example 10 μM vinblastine could completely abolish the enhancement in BisANS binding caused by 2 μM IKP104. In another experiment (not shown), we looked at the effect of different concentrations of vinblastine on the effect of IKP104. Since the increase in BisANS binding was roughly linear during the first 80 min of incubation at 37 °C, we assumed that the slope of the line that best fit the results obtained in that time period was an indication of the magnitude of the effect; in this experiment, we found that the enhancing effect of 10 μM IKP104 was inhibited 16% by 20 μM vinblastine, 25% by 50 μM vinblastine, and 40% by 100 μM vinblastine.

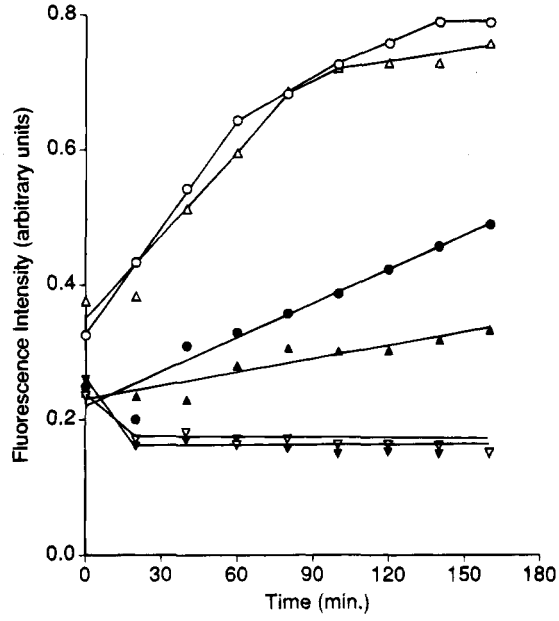

FIGURE 6: Effects of IKP104 on the binding of BisANS to tubulin and its modulation by vinblastine and phomopsin A. Samples of tubulin (0.208 mg/mL) were incubated at 37 °C in the absence (●) or presence of 10 μM concentrations of either IKP104 (○), vinblastine (▲), or phomopsin A (▼) alone or in the presence of 10 μM concentrations of both IKP104 and vinblastine (△) or both IKP104 and phomopsin A (▽). At the indicated times, 1-mL aliquots were removed, made 10 μM in BisANS, and placed in fluorescence cuvettes; the fluorescence was determined using the method described in Materials and Methods.

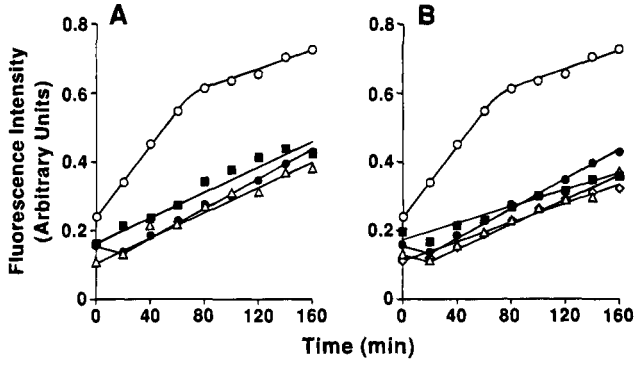

FIGURE 7: Modulation by podophyllotoxin and maytansine of the effect of IKP104 on the binding of BisANS to tubulin. (A) Samples of tubulin (0.208 mg/mL) were incubated at 37 °C in the absence (●) or presence of 10 μM concentrations of either IKP104 (○) or maytansine (△) or 10 μM concentrations of both IKP104 and maytansine together (■). (B) Samples of tubulin (0.208 mg/mL) were incubated at 37 °C in the absence (●) or presence of 10 μM concentrations of either IKP104 (○) or podophyllotoxin (△) or 10 μM concentrations of both IKP104 and podophyllotoxin (■) or both podophyllotoxin and maytansine (◇). At the indicated times, 1-mL aliquots were removed, made 10 μM in BisANS, and placed in fluorescence cuvettes; the fluorescence was determined using the method described in Materials and Methods.

As was the case with the sulfhydryl titers, maytansine was more effective at inhibiting the enhancing effect of IKP104 (Figure 7A); nevertheless, over the course of 160 min, 10 μM IKP104 was able to cause 29% ± 17% enhancement of BisANS binding in the presence of 10 μM maytansine (Figure 7A). A contrast to the effect of maytansine on IKP104 binding was provided in an experiment (not shown) in which 10 μM maytansine had no effect on the inhibitory effect of 10 μM colchicine; this is not surprising, since the

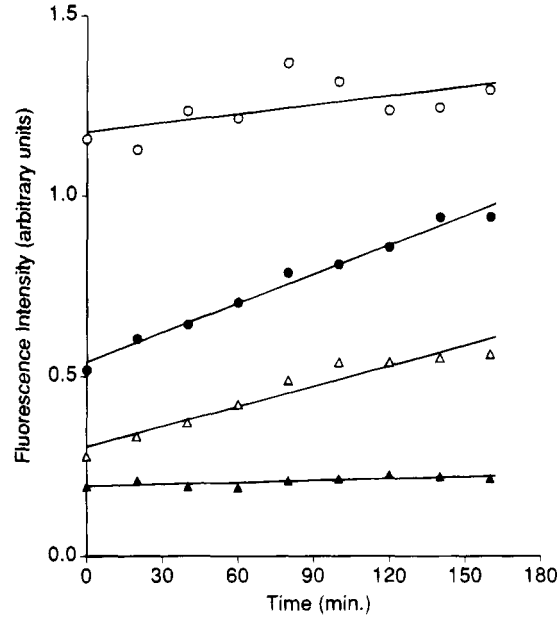

FIGURE 8: Modulation by colchicine of the effect of IKP104 on the binding of BisANS to tubulin. Samples of tubulin (0.208 mg/mL) were preincubated for 1 h at 37 °C in the absence (●) or presence of 10 μM concentrations of either IKP104 (○) or colchicine (△) or both IKP104 and colchicine (▲). After the preincubation was over, incubation continued at 37 °C. At the indicated times, 1-mL aliquots were removed, made 10 μM in BisANS, and placed in fluorescence cuvettes; the fluorescence was determined using the method described in Materials and Methods.

two drugs bind to different sites on the protein (Bhattacharyya & Wolff, 1977).

Podophyllotoxin is known to inhibit BisANS binding (Prasad et al., 1986). We found that IKP104 also counters the effect of podophyllotoxin; over the course of 160 min, 10 μM IKP104 caused 23% ± 18% enhancement of BisANS binding in the presence of 10 μM podophyllotoxin (Figure 7B). In contrast, 10 μM maytansine did not alter podophyllotoxin's suppression of BisANS binding (Figure 7B).

Interestingly, the most dramatic result was obtained with colchicine. In the presence of both IKP104 and colchicine, the binding of BisANS was actually less than that observed with either drug separately and showed no sign of increasing during the incubation (Figure 8). For the experiment in which the effects of colchicine and IKP104 were assayed, it was necessary to do a 1-h preincubation to allow time for the colchicine to bind. The results showed that colchicine inhibited binding of BisANS; as was previously shown, IKP104 showed the maximal enhancing effect because the 1-h preincubation allowed its conformation effect to be completed. However, the combination of colchicine and IKP104 gave strong inhibition of BisANS binding, much more than was seen with colchicine alone.

Effect of IKP104 on the Binding of [^{3}H]Colchicine to Tubulin. Tubulin ligands such as maytansine, which have no effect on either alkylation or BisANS binding, do not stabilize colchicine binding, whereas ligands such as vinblastine and phomopsin A, which inhibit both alkylation and BisANS binding, strongly stabilize colchicine binding. One would therefore predict that IKP104, which enhances alkylation and BisANS binding, should accelerate the decay of colchicine binding to tubulin. As shown in Figure 9, this is indeed the case. Tubulin incubated at 37 °C in the absence of IKP104 lost its ability to bind to colchicine with a half-

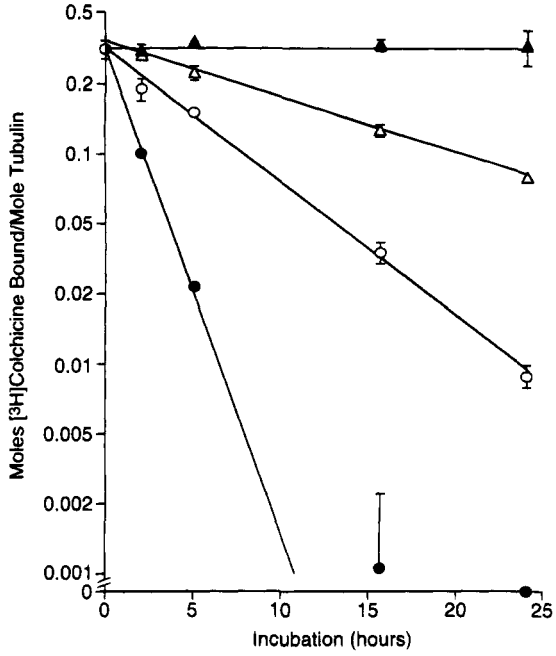

FIGURE 9: Effect of IKP104 on the binding of [^{3}H]colchicine to tubulin. Samples of tubulin (0.3 mg/mL) were incubated at 37 °C as follows: one set of samples was incubated in the absence of [^{3}H]colchicine and in the presence (●) or absence (○) of 20 μM IKP104. At the indicated times, triplicate aliquots (200 μL) were removed, made 9 μM in [^{3}H]colchicine (338 Ci/mol), and incubated for a further 2 h and then the amount of [^{3}H]colchicine bound to tubulin was determined. Another set was incubated in the presence of 10 μM [^{3}H]colchicine (338 Ci/mol) and in the presence (▲) or absence (△) of 20 μM IKP104. At the indicated times, triplicate aliquots (200 μL) were removed and the binding of [^{3}H]colchicine to the tubulin was determined. Triplicate aliquots were taken for each time point except for the sample at 0 h, lacking IKP104, not preincubated with [^{3}H]colchicine, from which duplicate aliquots were taken. Standard deviations are shown.

time of 4.6 h. In the presence of IKP104, the loss was much faster, with a half-time of 1.25 h. Surprisingly, however, when tubulin was incubated with [^{3}H]colchicine from the beginning, IKP104 stabilized the binding of colchicine. As seen in Figure 9, tubulin incubated at 37 °C with [^{3}H]-colchicine lost the bound colchicine with a half-time of 11.5 h; however, if IKP104 was present, there was no measurable loss over a period of 24 h. In other experiments (not shown) in which the loss of bound [^{3}H]colchicine at 37 °C was measured over a 3-day period, the half-time of decay in the absence of IKP104 was 9.3−11.2 h but was 49−66 h in the presence of IKP104.

It appeared from this experiment and the one shown in Figure 8 that IKP104 and colchicine together gave extra stability to the tubulin molecule; this results appears to be inconsistent with the observation that IKP104 and colchicine, in combination, did not appear to inhibit alkylation of tubulin sulfhydryls more than did colchicine alone (Figure 5A). However, when tubulin was preincubated with colchicine for 1 h at 37 °C prior to alkylation, we found that the alkylation of tubulin was inhibited by IKP104 and colchicine in combination more than by colchicine alone even though IKP104 by itself enhanced alkylation (Table 1).

DISCUSSION

The binding of IKP104 to tubulin is complex. The kinetics of binding were most consistent with two classes of binding

Table 1: Effects of IKP104 and Colchicine on the Alkylation of Tubulin[a]

addition at 0 min	addition at 60 min	mol of ^{14}C/ mol of tubulin	% of control
none	DMSO	3.81 ± 0.10	100
none	IKP104	6.24 ± 0.36	164
colchicine	DMSO	2.36 ± 0.05	62
colchicine	IKP104	1.63 ± 0.07	43

[a] Aliquots (240 μL) of tubulin (0.69 mg/mL) were incubated for 60 min at 37 °C in the presence or absence of 21 μM colchicine. After 60 min, 5 μL of IKP104 (dissolved in DMSO) was added to half of the aliquots and 5 μL of DMSO to the other half. All aliquots were then made 1.36 mM in iodo[^{14}C]acetamide (0.66 Ci/mol). Final concentrations of tubulin, IKP104, and colchicine were, respectively, 0.66 mg/mL, 20 μM, and 20 μM. Incorporation of ^{14}C label was determined as described in Materials and Methods. Each combination was done in quadrupole aliquots. Standard deviations are shown.

site, differing in affinity; the values of [$D_{0.5}$] for the high- and low-affinity sites were, respectively, 0.21 ± 0.09 μM and 17 ± 4 μM. These are not the same as the actual K_d values for the two sites, but the fact that they differ by 2 orders of magnitude suggests that the actual K_d's may differ considerably as well. Interestingly, the effect on fluorescence was much greater for the low-affinity than for the high-affinity sites. This may reflect that IKP104 binds differently at the two sites. Perhaps the fluorophore in IKP104 is less constrained at the high-affinity site than at the other site and hence fluoresces less. It is also possible that there are a greater number of low-affinity binding sites. The two-site model is consistent with the observation that removal of the C-terminal region of α- and β-tubulin by subtilisin almost abolishes the low-affinity site, yielding one type of site with an apparent K_d of 0.14 μM (Chaudhuri and Ludueña, unpublished results). Since IKP104 is known to induce tubulin aggregation (Mizuhashi et al., 1992), it is conceivable that the low-affinity binding could reflect this aggregation (Na & Timasheff, 1986). The fact that most of this binding is eliminated by subtilisin digestion, however, suggests otherwise, since subtilisin-digested tubulin is more susceptible to vinblastine-induced aggregation (Serrano et al., 1986) and, by analogy, might also be more susceptible to IKP104-induced aggregation.

Consistent with the complex binding, IKP104 appeared to have an unusual constellation of effects on the tubulin molecule. In its presence, formation of both the β^s and β^* cross-links was inhibited. A specific inhibition of cross-link formation, however, should be accompanied by an increase in the yield of non-cross-linked β-tubulin. As seen in Figure 3, this was true for IKP104's inhibition of β^s formation but not for its inhibition of β^* formation. In the latter case, raising the concentration of IKP104 caused increased inhibition of β^* formation, while the yield of non-cross-linked β_1 decreased and that of non-specifically cross-linked aggregated β_1 increased greatly. In short, it appeared that IKP104's inhibition of β^* formation was explicable as a result of its unusually large enhancement of the exposure of sulfhydryl groups on the surface of the tubulin molecule, which would allow many competing cross-linking reactions to occur, thereby diminishing the yield of both the β^* band and the residual non-cross-linked β_1 band. In contrast, IKP104's inhibition of β^s formation appeared to be a specific effect; hence, IKP104 should be classified in the category of tubulin ligands that includes vinblastine, phomopsin A,

ustiloxin A, dolastatin 10, maytansine, rhizoxin, halichondrin B, and homohalichondrin B rather than in any other category. This is what was proposed by Mizuhashi et al. (1992) on the basis of their observation that IKP104 induces tubulin to polymerize into spirals similar to those seen in the presence of vinblastine.[2]

Placing IKP104 into the category of ligands which binds in the vinblastine domain of tubulin may at first glance appear to contradict the results of the experiments shown in Figure 5, in which the enhancing effect of IKP104 on tubulin decay can be partly or completely blocked by appropriate concentrations of colchicine or podophyllotoxin, which do not bind in this domain, as well as by phomopsin A, vinblastine, and maytansine, which do bind here. In interpreting the results shown in Figure 5, we must remember that a ligand may counter the effect of IKP104 on tubulin either by inhibiting the binding of IKP104 to tubulin or by binding elsewhere on the tubulin molecule and exerting a conformational effect which modifies or even overrides that of IKP104. The fact that 100 μM IKP104 could fully abolish the effect of 10 μM vinblastine and partly abolish that of 10 μM maytansine is more consistent with the hypothesis that vinblastine and perhaps maytansine compete with IKP104 for binding to tubulin, but the inability of high concentrations of IKP104 to override the effects of colchicine, podophyllotoxin, or phomopsin A is consistent with either hypothesis.

The most unusual effect of IKP104 was its pronounced enhancement of the rate of decay of tubulin as measured by the exposure of sulfhydryl groups or hydrophobic areas in the surface of the tubulin molecule; the magnitude of this enhancement was on the order of 100−200%. This effect was also apparent by the classical assay in which preincubation with IKP104 greatly accelerated the loss of tubulin's colchicine-binding activity. As seen in Figure 9, for example, IKP104 preincubation shortened the half-time of decay by 73%. IKP104 did not simply accelerate the rate of decay; it appeared to complete the process. Whatever the nature of the process of tubulin decay, the process appeared to attain completion in the presence of IKP104. As can be seen in Figure 4, exposure of sulfhydryl groups seemed to reach a maximum before 10 h, beyond which no more exposure took place. Exposure of hydrophobic areas did not reach a maximum but the rate decreased significantly after 60−80 minutes. Other tubulin ligands which we have studied either had no effect on decay or else slowed down the process. The exception is halichondrin B, which accelerates the rate of exposure of hydrophobic areas; even with this ligand, however, there was no suggestion that a maximum was reached (Luduena et al., 1993). As seen in Figure 4, however, the decay of tubulin in the absence of ligand did not attain a maximum even after 48 h of incubation.

A simple explanation of these results is that IKP104 induces the tubulin molecule to enter a conformational state very different from that observed in the presence of ligands such as vinblastine or phomopsin A, which inhibit exposure of sulfhydryl groups and hydrophobic areas. We have previously observed that, in the presence of colchicine and phomopsin A in combination, tubulin decay appears to be almost completely arrested (Luduena et al., 1990). The conformational change induced by IKP104 may involve a partial unfolding of the tubulin molecule.

Perhaps the most unexpected observation in these experiments is that, in the presence of colchicine, IKP104 appeared to stabilize tubulin almost completely. When tubulin was incubated with both [^{3}H]colchicine and IKP104, loss of bound [^{3}H]colchicine was very slow (Figure 9). Similarly, both colchicine and podophyllotoxin could inhibit IKP104's enhancement of exposure of sulfhydryl groups and hydrophobic areas. One possible explanation is that IKP104 induces a conformational change restricted to that region of the tubulin molecule to which colchicine binds; IKP104 may actually stabilize the region around its own binding site. By this model, the conformational change induced by IKP104 would destabilize that portion of the tubulin and greatly enhance the rate of exposure of sulfhydryl groups and hydrophobic areas. If colchicine is present, however, then it would bind in this region and stabilize it against decay. Another possibility arises from the observation that IKP104 appears to have two binding sites on tubulin (Figure 2); perhaps the binding of IKP104 to one site stimulates decay while binding to the other inhibits decay. If this is the case, then perhaps, in the absence of other ligands, the effect enhancing decay is dominant, whereas colchicine might abolish the effect of IKP104 at that site while permitting the effect at the stabilizing site to occur.

The tubulin molecule may have multiple conformational states accessible to it. IKP104 may induce tubulin to shift into one of these states. Since tubulin in the presence of vinblastine can form spiral protofilaments which bind to colchicine and podophyllotoxin (Luduena et al., 1986b; Palanivelu & Luduena, 1982), we have previously argued that the conformational state of tubulin in the presence of certain ligands may resemble that of tubulin in the intact microtubule. However, these protofilaments are spirals and not straight as they are in microtubule; hence, their conformation may be different. The conformational state observed in the presence of colchicine and IKP104 together appears to be very stable. Perhaps it is a different state from the stable one obtained in the presence of colchicine and phomopsin A together (Luduena et al., 1990). The conformational state of tubulin attained after 10 h of incubation with IKP104 also appears to be very stable (Figure 4). This is a state in which many sulfhydryl groups and hydrophobic areas are exposed; undoubtedly this state is different from the one stabilized by phomopsin A, in which few sulfhydryl groups and hydrophobic areas not exposed (Luduena et al., 1990).

It is possible that the tubulin molecule undergoes conformational changes during microtubule assembly. Whether one change occurs at the moment of addition to the microtubule or subsequently when the GTP is hydrolyzed, or at both times, is not clear. A full understanding of the mechanism of microtubule assembly would require a knowledge of the three-dimensional structure of tubulin in each of its conformational states. For this purpose, ligands which stabilize the tubulin molecule in any state would be very

[2] Batra et al. (1986) examined a series of derivatives of 5,6-diphenylpyridazin-3-one and found that several of them, particularly those with chlorine substituents on the phenyl rings, were excellent inhibitors of microtubule assembly *in vitro*. However, unlike the structurally related IKP104, none of these were able to inhibit vinblastine binding to tubulin. It is possible that the additional phenyl ring on IKP104 as well as the methoxy substituents, which are even bulkier than chlorines, on the phenyl rings may cause the IKP104 binding site to overlap with that of vinblastine.

useful. If IKP104 can stablize a conformational state different from that stabilized by other ligands, then it could be an extremely valuable tool for understanding the properties of the tubulin molecule and the mechanism of microtubule assembly, perhaps by using electron crystallography to determine the structure of tubulin in zinc-induced sheets in the presence of IKP104, as has recently been done in the presence of taxol (Nogales et al., 1995).

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