IKP104-Induced Decay of Tubulin: Role of the A-Ring Binding Site of Colchicine†

Asish R. Chaudhuri,*,‡ Isao Tomita,§ Fukutaro Mizuhashi,|| Kyoji Murata,|| James L. Potenziano,⊥ and Richard F. Ludueña‡

Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284, Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan, Kumiai Chemical Industry Company, Tokyo, Japan, and Department of Chemistry, State University of New York at Binghamton, Binghamton, New York 13902-6000

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ABSTRACT: Tubulin, the major subunit protein of microtubules, has a tendency to lose its ability to assemble or to interact with ligands in a time-dependent process known as decay. Decay involves the increase in exposure of sulfhydryl groups and hydrophobic areas. The antimitotic drug IKP104 [2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone] accelerates the decay of tubulin [Ludueña et al. (1995) *Biochemistry 34,* 15751−15759]. In the presence of colchicine, however, IKP104 stabilizes tubulin against decay. We have shown that the stability and the acceleration of the decay of tubulin are mediated respectively by the high- and low-affinity binding site(s) of IKP104 [Chaudhuri et al. (1998) *J. Protein Chem. 17,* 303−309]. To better understand the mechanism by which colchicine protects tubulin from IKP104-induced decay, we examined the effect of colchicine and its analogues on this process. We found that IKP104 unfolds tubulin in a process involving a specific domain where colchicine interacts, although the binding sites of these two drugs are distinctly different. 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropolone (MTPT), the bicyclic analogue of colchicine that lacks the B-ring, can also protect tubulin from IKP104-induced decay. An A-ring analogue of colchicine, 3,4,5-trimethoxybenzaldehyde (TMB), can also stop IKP104-induced unfolding of tubulin significantly. Interestingly, the C-ring analogue of colchicine, tropolone methyl ether (TME), does not prevent this process. Our results thus suggest that neither the B-ring nor the C-ring binding regions of colchicine are involved in the IKP104-induced decay and that the A-ring binding site of colchicine on tubulin plays a crucial role in IKP104-induced decay.

Microtubules are ubiquitous cellular organelles playing crucial roles in mitosis, motility, transport, and other processes (*1*). They are composed of tubulin, an $\alpha\beta$ heterodimer with an unusual set of conformational properties.

Tubulin is thought to undergo a conformational change in the process of microtubule assembly and subsequent hydrolysis of the bound GTP (*2*). In addition, tubulin has long been known to undergo a time-dependent loss of its ability to assemble and to bind to ligands, a process known as decay, during which sulfhydryl groups and hydrophobic areas become exposed (*3*). The mechanism of this process is still not clear; however, it is possible that the conformational changes involved in assembly and in decay may be related to one another and that an examination of the mechanism of decay may be useful in understanding assembly.

Tubulin decay is strongly influenced by ligands, although these effects are very variable. Ligands such as colchicine and vinblastine stabilize against decay; in contrast, halichondrin B accelerates decay. Certain ligands, such as maytansine, have no effect on decay. One of the most interesting and complex of these ligands is IKP104[1] (Figure 1); this compound greatly enhances decay, as measured by loss of colchicine binding and exposure of sulfhydryl groups and hydrophobic areas (*3*). Paradoxically, however, if tubulin is preincubated with colchicine, podophyllotoxin, or nocodazole, then IKP104 becomes a strong inhibitor of decay, much stronger than any of these drugs alone (*3, 4*).

We have here attempted to dissect these competing effects of colchicine and IKP104 using colchicine and colchicine analogues lacking one or two of the three rings of colchicine. First, we found that IKP104-induced unfolding of tubulin initiates from a specific domain where colchicine interacts, although the binding sites of these two drugs are distinctly different. Second, we have found that 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl) tropolone (MTPT), which lacks the B-ring, has the same decay-inhibiting effect as colchicine. The C-ring analogue tropolone methyl ether (TME) does not inhibit IKP104's decay-enhancing effect. Interestingly, the

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* Address correspondence to this author at the Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760. Tel (210) 567-3731; FAX (210) 567-6595.

‡ University of Texas Health Science Center.

§ University of Shizuoka.

|| Kumiai Chemical Industry Co.

⊥ SUNY−Binghamton.

[1] Abbreviations: IKP104, 2-(4-fluorophenyl)-1-(2-chloro-3,5-dimethoxyphenyl)-3-methyl-6-phenyl-4(1*H*)-pyridinone; MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropolone; TMB, 3,4,5-trimethoxybenzaldehyde; TME, tropolone methyl ether; bisANS, bis(8-anilinonaphthalene-1-sulfonate); DMSO, dimethyl sulfoxide; EBI, *N*,*N*′-ethylenebis(iodoacetamide).

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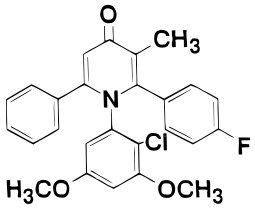

FIGURE 1: Structure of IKP104.

A-ring analogue 3,4,5-trimethoxybenzaldehyde (TMB) inhibits the decay-enhancing effect of IKP104 significantly, as determined by measuring the exposure of hydrophobic areas and sulfhydryl groups. These results therefore suggest that the key target on tubulin for IKP104's conformational effects is the region where the A-ring of colchicine binds.

EXPERIMENTAL PROCEDURES

Materials. IKP104 was produced at the K−I Research Institute (Shizuoka, Japan); TMB and bisANS were purchased from Aldrich Chemical Co. and Molecular Probes (Junction City, OR), respectively. MTPT was the kind gift of Dr. Thomas J. Fitzgerald, Florida Agricultural and Mechanical University, Tallahassee, FL. TME was synthesized as described previously (*5*). Sources of all other materials were as previously described (*6*). IKP104 was dissolved in DMSO immediately prior to use.

Tubulin Preparation. Microtubules were purified from bovine cerebra by recycling and tubulin was purified therefrom by phosphocellulose chromatography (*7*). Experiments were done in the following buffer: 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.4, 1 mM ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (*7*).

Alkylation Reactions. In most experiments, tubulin was reacted with iodo[^{14}C]acetamide for 2 h at 37 °C. After the reactions, tubulin samples were precipitated with 10% trichloroacetic acid, the precipitates were collected by filtration, and the radioactivity of the filters was determined (*8*). In some experiments, the reaction with iodo[^{14}C]-acetamide was measured by subjecting the reduced and carboxamidomethylated protein to polyacrylamide gel electrophoresis and then staining, scanning, and slicing the gel and counting the slices (*9*).

Fluorescence. Samples of tubulin containing the drug were incubated at 37 °C. At periodic intervals, aliquots were withdrawn, made 10 μM in bisANS, and examined in a Hitachi model F-2000 spectrofluorometer. Excitation and emission were at 385 and 490 nm, respectively. The quantum yield of the tubulin−colchicine complex is negligible compared to the fluorescence quantum yield of the tubulin−bisANS complex at 490 nm, when excited at 385 nm.

For measurement of the binding of IKP104 to tubulin, aliquots of tubulin (1 μM) preincubated with colchicine (10 μM) at 37 °C for 1 h were incubated in the presence of different concentrations of IKP104 (0−9 μM) at 37 °C for 30 min. Excitation and emission maxima of IKP104 were at 278 and 467 nm, respectively. In the presence of tubulin, the emission maximum of IKP104 shifted from 467 to 451 nm with the increment of fluorescence intensity also shifting. The fluorescence at 451 nm of the same series of concentrations of IKP104 with a fixed concentration of colchicine (10 μM), in the absence of tubulin, was also measured. The fluorescence of all the samples was corrected for the inner-filter effect according to Lakowicz (*10*). There is no potential energy transfer between IKP104 and tubulin because IKP104 does not have any absorbance at 335 nm where tubulin fluoresces, and similarly, tubulin does not absorb at 467 nm where IKP104 fluoresces. Moreover, there is also no energy transfer between IKP104 and the tubulin−colchicine complex. IKP104 does not have any absorbance between 300 and 350 nm where the tubulin−colchicine complex gives the maximum fluorescence (tryptophan fluorescence) when excited at 278 nm. Tubulin or the tubulin−colchicine complex does not fluoresce at 451 nm when excited at 278 nm. The difference in fluorescence intensity (ΔF) between tubulin−colchicine−IKP104 and colchicine−IKP104 at different concentrations of IKP104 at 451 nm was measured and the fluorescence values (ΔF) of the binding of IKP104 to the tubulin−colchicine complex were analyzed because of a two-site model equation as follows:

$$F_1 = F_{m1}D/(K_{d1} + D)$$

$$F_2 = F_{m2}D/(K_{d2} + D)$$

$$F = F_1 + F_2$$

where F_1 and F_2 are the observed corrected fluorescence values at any drug concentration D for high- and low-affinity sites, respectively; F_{m1} and F_{m2} are the maximum fluorescence values for high- and low-affinity sites, respectively; K_{d1} and K_{d2} are the apparent dissociation constants for high- and low-affinity sites, respectively; and F is the total fluorescence value at any drug concentration. The data were fitted to a two-site model equation using nonlinear curve-fitting software MINSQ, version 3.2 (Micromath Scientific Software, Salt Lake City, UT) as described (*3, 11*).

Other Methods. The binding of [^{3}H]colchicine (50 μM) to tubulin (5 μM) was measured by filtration with DEAE-cellulose filters (*12*). The concentration used for both TMB and TME was 5 mM. Time and temperature of incubation were, respectively, 1 h and 37 °C. Each combination was in duplicate. Protein concentrations were determined by a modified form of the procedure of Lowry et al. (*13*) with bovine serum albumin as a standard (*14*).

RESULTS

We previously showed that, in the presence of colchicine (*3*), only the decay-inhibiting property of IKP104 was expressed. We have attempted here to dissect this phenomenon by identifying the domain of the colchicine binding site on tubulin that causes the effect.

As we showed before (*3, 11*), IKP104 has two classes of binding sites on tubulin. The stoichiometries of binding at the two classes of site have not been ascertained. However, the apparent dissociation constants for the high- and low-affinity sites are, respectively, $K_{d1} = 0.21 \pm 0.09$ μM and $K_{d2} = 17 \pm 4$ μM (*3, 11*). To see the effect of colchicine on the binding of IKP104 to tubulin, we studied the binding of IKP104 to the tubulin−colchicine complex fluorometrically. We found that colchicine does not alter the biphasic nature of binding of IKP104 (Figure 2) to tubulin. The apparent K_d values for the high- and low-affinity binding sites for IKP104

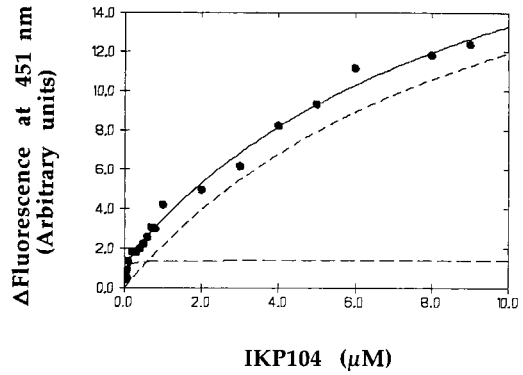

FIGURE 2: Analysis of the binding of IKP104 to the colchicine−tubulin complex. Aliquots of tubulin−colchicine (1 μM tubulin preincubated with 10 μM colchicine at 37 °C for 1 h) were incubated with a series of concentrations of IKP104 (0−9 μM) at 37 °C for 30 min and then excited at 278 nm. Emission at 451 nm was measured. The emission at 451 nm of the same series of concentrations of IKP104 containing colchicine (10 μM), in the absence of tubulin, was also measured. The observed fluorescence values were corrected for the inner filter effect. The differences of fluorescence values (ΔF) between tubulin-colchicine-IKP104 and colchicine-IKP104 at 451 nm were fitted to a two-site model by using nonlinear curve fitting software MINSQ as mentioned in "Experimental Procedures". The hypothetical curves for each type of binding site are shown as dashed lines. The lower and the upper dashed lines correspond to high- and low-affinity sites, respectively.

Table 1: Modulation by Colchicine of the Effect of IKP104 on the Alkylation of Tubulin[a]

addition	mol of ^{14}C/mol of tubulin
none	3.89 ± 0.10
IKP104	5.37 ± 0.02
colchicine	3.22 ± 0.04
colchicine + IKP104	2.05 ± 0.21

[a] Aliquots (500 μL) of tubulin (0.5 mg/mL) were preincubated at 37 °C for 45 min in the presence or absence of 50 μM colchicine. After 45 min, samples were made 20 μM in IKP104 by addition of 10 μL of 1 mM IKP104, dissolved in DMSO, and incubated at 37 °C for 30 min. To the control sample was added 10 μL of DMSO. After the incubation, 3.66 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 °C was continued for 2 h. Incorporation of ^{14}C was determined as described under Experimental Procedures. Each combination was done in triplicate. Standard deviations are shown.

on tubulin in the presence of colchicine are, respectively, 0.04 μM and 11 μM. The low-affinity site binding value is comparable to the value of 17 ± 4 μM obtained in the absence of colchicine (*3*, *11*), while the affinity of the high-affinity site of IKP104 is increased approximately 3-fold in the presence of colchicine.

Since the sulfhydryl groups of tubulin are sensitive reporters for studying its conformation and its interactions with drugs (*15*), we measured the effect of IKP104 on the sulfhydryl titer of tubulin and also measured the modulation by colchicine of the effect of IKP104 on tubulin. Table 1 shows that IKP104 increases the sulfhydryl titer of tubulin by approximately 1.5 mol/mol, as was previously noted (*11*). In contrast, in the case of the tubulin−colchicine complex, IKP104 decreases the sulfhydryl titer by approximately 1 mol/mol instead of increasing the titer. When we examined the effects of these drugs on the alkylation of the individual α and β subunits, we found that IKP104, in the absence of colchicine, increases the sulfhydryl titer of α and β by 1.16 and 0.98 mol/mol, respectively (Table 2). In the presence of colchicine (Table 2), however, IKP104 lowers the sulfhydryl titer of α and β by 1.67 and 1.38 mol/mol, respectively. We previously observed that the sulfhydryl titer of tubulin varied from one preparation to another (*16*, *17*). Comparison of Tables 1 and 2 shows that this is true. The important point, however, is that with each batch of tubulin, IKP104, in the absence of colchicine, increases the sulfhydryl titer and, in the presence of colchicine, decreases the sulfhydryl titer of both the α and β subunits.

Table 2: Modulation by Colchicine of the Effect of IKP104 on the Alkylation of the α and β Subunits of Tubulin[a]

additions	mol of ^{14}C/mol of subunit
none	α 4.65 ± 0.17
	β 3.73 ± 0.10
IKP104	α 5.81 ± 0.07
	β 4.71 ± 0.14
colchicine	α 3.60 ± 0.03
	β 3.20 ± 0.07
colchicine + IKP104	α 1.93 ± 0.10
	β 1.82 ± 0.16

[a] Aliquots (500 μL) of tubulin (0.5 mg/mL) were preincubated at 37 °C for 45 min in the presence or absence of 50 μM colchicine. After 45 min, samples were made 20 μM in IKP104 by addition of 10 μL of 1 mM IKP104, dissolved in DMSO and incubated at 37 °C for 30 min. To the control sample was added 10 μL of DMSO. After the 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 °C was continued for 2 h. The samples were dialyzed against buffer, reduced, carboxamidomethylated, and were 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 proteins. Gels were sliced, and the radioactivity of the slices was determined. Each combination was done in triplicate. Standard deviations are shown.

To dissect the interaction of the conformational effects on tubulin of colchicine and IKP104, we used three analogues of colchicine. One, MTPT, a bicyclic analogue of the A- and C-rings of colchicine, is a well-known inhibitor of colchicine binding to tubulin (*18*). The other two compounds, the A-ring analogue TMB and the C-ring analogue TME, have been shown to inhibit colchicine binding to tubulin (*19*) and indeed do so at the concentrations and under the conditions used in our experiments, where 5 mM concentrations of TMB and TME inhibit the binding of [^{3}H]colchicine (50 μM) to tubulin by 30% ± 2% and 18% ± 3%, respectively (data not shown). The K_d values of TMB and TME are reported as 2.5 mM and 0.45 mM, respectively, considerably weaker than colchicine's K_d of 0.3 μM (*20*).

We examined the effects of each of these analogues on bisANS binding to tubulin (Table 3). As is the case with colchicine, MTPT inhibits bisANS binding, although the magnitude of the inhibition is much less than for colchicine (*21*); addition of IKP104 causes still more inhibition. The A-ring analogue TMB by itself has no effect on bisANS binding but strongly blocks the enhancement caused by IKP104; in the presence of TMB, IKP104-induced enhancement of bisANS binding is only 37% as much as that observed in the absence of TMB. In contrast, TME has very little modulatory influence on IKP104's effect. TME, by itself, inhibits bisANS binding by 10%; when IKP104 is added, however, bisANS binding is enhanced by 90%, not very different from the 101% increase observed when IKP104 is added in the absence of TME. Another way of

Table 3: Modulation by MTPT, TMB, and TME of the Effect of IKP104 on BisANS Binding to Tubulin[a]

samples	fluorescence at 490 nm (arbitrary units)	% of control
tubulin	53.57 ± 2.83	100
tubulin + IKP104	107.80 ± 0.26	201
tubulin + MTPT	49.20 ± 0.25	92
tubulin + MTPT + IKP104	37.13 ± 0.12	69
tubulin + TMB	53.09 ± 0.99	99
tubulin + TMB + IKP104	73.03 ± 1.35	136
tubulin + TME	48.08 ± 0.77	90
tubulin + TME + IKP104	91.23 ± 0.17	170

[a] Aliquots (500 μL) of tubulin (0.2 mg/mL) were preincubated at 37°C for 45 min in the absence or presence of MTPT (50 μM), TMB (5 mM), and TME (5 mM) followed by incubation with IKP104 (20 μM) at 37°C for another 30 min. To the control samples, an equal volume of DMSO was added. After the incubation was over, samples were mixed with 5 μL of bisANS (to give a final concentration of 10 μM). The samples were placed in a fluorescence cuvette and fluorescence intensity was measured. Excitation and emission were at 385 and 490 nm, respectively. Each combination was in triplicate. Standard deviations are shown.

Table 4: Modulation by Colchicine and MTPT of the Effect of IKP104 on the Alkylation of Tubulin

additions	mol of ^{14}C/mol of tubulin
none	3.73 ± 0.19
IKP104	4.65 ± 0.01
colchicine	2.78 ± 0.10
colchicine + IKP104	1.82 ± 0.02
MTPT	3.65 ± 0.15
MTPT + IKP104	2.43 ± 0.04

[a] Aliquots (500 μL) of tubulin (0.5 mg/mL) were preincubated at 37 °C for 45 min in the absence or presence of colchicine (50 μM) or MTPT (50 μM). After 45 min, the samples were made 20 μM in IKP104 by addition of 10 μL of 1 mM IKP104, dissolved in DMSO, and incubated at 37 °C for 30 min. To the control sample was added an equal volume of DMSO. After the incubation, 3.66 μL of iodo[^{14}C]acetamide (0.58 Ci/mol) was added to each sample to a final concentration of 500 μM. The incubation was continued for 2 h. Incorporation of ^{14}C was determined as described under Experimental Procedures. Each combination was in triplicate. Standard deviations are shown.

presenting these data is to say that TME inhibits bisANS binding by 10% in the absence, and by 15% in the presence, of IKP104. These results suggest that the binding of the A-ring analogue TMB inhibits the conformational effect of IKP104 much more strongly than does the binding of the C-ring analogue TME.

We have found previously (*15*) that there is usually a very strong correlation between the effects of a ligand on bisANS binding to tubulin and its effects on exposure of sulfhydryl groups on tubulin. Accordingly, we investigated the interaction of IKP104 and the colchicine analogues on the alkylation of tubulin sulfhydryls by iodo[^{14}C]acetamide (Tables 4 and 5). We found that colchicine inhibited alkylation by 25%. In the presence of IKP104 and colchicine together, alkylation was inhibited by 51%. Although MTPT by itself had little or no effect on alkylation, MTPT and IKP104 together inhibited alkylation by 25% (Table 4). These effects are very similar to those observed with bisANS binding.

The C-ring analogue TME, by itself, has no effect on alkylation; TME also has no effect at all on IKP104's enhancement of alkylation (Table 5). The effect of the A-ring analogue TMB is more difficult to interpret since TMB by itself strongly enhances alkylation (by 26%). Addition of

Table 5: Modulation by A- and C-Ring Analogues of the Effect of IKP104 on the Alkylation of Tubulin[a]

additions	mol of ^{14}C/mol of tubulin
none	2.90 ± 0.01
IKP104	4.06 ± 0.24
TMB	3.66 ± 0.08
TMB + IKP104	4.03 ± 0.07
TME	2.90 ± 0.14
TME + IKP104	4.07 ± 0.10

[a] Aliquots (500 μL) of tubulin (0.5 mg/mL) were preincubated at 37 °C for 45 min in the absence or presence of TMB (5 mM) and TME (5 mM). After 45 min, the samples were made 20 μM in IKP104 by addition of 10 μL of 1 mM IKP104, dissolved in DMSO, and incubated at 37 °C for 30 min. To the control sample was added an equal volume of DMSO. After the incubation, 3.66 μL of iodo[^{14}C]acetamide (0.58 Ci/mol) was added to each sample to a final concentration of 500 μM. The incubation was continued for 2 h. Incorporation of ^{14}C was determined as described under Experimental Procedures. All the combinations other than the underlined ones were in triplicate. The underlined samples were done in sets of 10. Standard deviations are shown.

IKP104 causes further enhancement to a level indistinguishable from that observed when IKP104 is added in the absence of TMB. In view of the results obtained with bisANS, one simple interpretation of these results is that TME does not interfere with the conformational effect of IKP104, whereas TMB's effect competes with that of IKP104. By this analysis, we would say that, in the absence of any ligand, IKP104 enhances alkylation by 40%; in the presence of TME, IKP104 also enhances alkylation by 40%. In contrast, in the presence of TMB, IKP104 enhances alkylation by only 10%.

Since IKP104 inhibits the exposure of the sulfhydryl groups of tubulin markedly in the presence of colchicine, we studied the modulation by different concentrations of colchicine on the effect of IKP104 on the exposure of the hydrophobic areas of tubulin as measured by bisANS binding. We found that prior incubation of tubulin with different concentrations of colchicine leads to more stable complexes in the presence of IKP104 (Figure 3).

DISCUSSION

The tubulin molecule has the unique property of binding with high affinity to a variety of very different drugs. Despite the fact that most of these drugs inhibit microtubule assembly, their effects on the conformation of tubulin are very variable. Some drugs, such as colchicine, podophyllotoxin, vinblastine, phomopsin A, ustiloxin A, and dolastatin 10 inhibit tubulin decay. Other drugs, such as maytansine and rhizoxin, have no effect on decay, while halichondrin B enhances decay (*8*, *21*−*27*). Of the tubulin-binding drugs, the one with the most complex effects on tubulin conformation is IKP104. Alone, it is a more effective enhancer of tubulin decay than any other ligand. In combination with colchicine, however, IKP104 inhibits decay almost as strongly as any other single ligand and much more strongly than does colchicine by itself (*3*). We have found that the apparently double effect of IKP104 is due to two classes of binding site; the low-affinity class of site enhances and the high-affinity class inhibits decay (*11*).

In the work reported here, we have attempted to dissect the nature of the mechanism by which colchicine turns IKP104 into a strong inhibitor of tubulin decay. We have

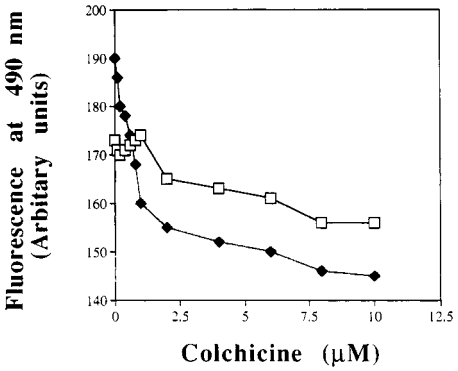

FIGURE 3: Modulation by different concentrations of colchicine of the effect of IKP104 on bisANS binding to tubulin. Aliquots of tubulin (0.2 mg/mL) were preincubated at 37 °C for 45 min in the presence of different concentrations of colchicine (0−10 μM). After that, IKP104 was added to a final concentration of 10 μM (◆) and the sample was incubated for another 30 min at 37 °C. To the control (□) was added an equal volume of DMSO. Aliquots (1 mL) were removed from each sample and mixed with 5 μL of bisANS (to give a final concentration of 10 μM), and the fluorescence was measured at 490 nm. Excitation was at 385 nm. (G: Tubulin + colchicine + DMSO; (◆) tubulin + colchicine + IKP104).

found that this synergistic effect embraces both the α and β subunits (Table 2). This suggests that the effect may involve a fairly substantial change in tubulin conformation. Clearly, the interaction of colchicine at its binding site must somehow influence the conformational effect of IKP104. There are, in principle, two ways to explain this. One possibility is that the low-affinity site (or sites) of IKP104, which accelerates the decay of tubulin (*11*), may share the same binding site with colchicine. If colchicine occupies its binding domain first, then IKP104 does not have a chance to interact with its low-affinity site and hence IKP104 cannot unfold tubulin. The other possibility is that the low-affinity binding site(s) of IKP104 could be distinctly different from the colchicine binding site, but upon interaction with tubulin, the low-affinity site of IKP104 unfolds a specific domain of tubulin where colchicine and its related drugs interact. If that crucial domain is already occupied by colchicine, IKP104 cannot unfold tubulin and as a result, the combined effects of IKP104 and colchicine stabilize tubulin more than any one of these drugs does. Our fluorescence binding results suggest that the second possibility is true. In the presence of colchicine, IKP104 binds to tubulin at two classes of site, differing in affinity (Figure 2), just as it does in the absence of colchicine (*3, 11*), although the affinity of IKP104 is increased at the high-affinity site. Interestingly, the fluorescence quantum yield generated by the low binding affinity site(s) of IKP104 is greater than that of the high-affinity site(s), as reported earlier (*3, 11*). This could be explained either by the IKP104 fluorophore being less constrained in the high-affinity site or by there being more low-affinity binding sites than high-affinity sites. Whatever the explanation is, our data suggest that the binding sites of IKP104 are distinctly different from the binding site of colchicine (Figure 2). The higher affinity of IKP104 at its high-affinity site is probably due to a conformational effect by colchicine on that domain. However, the alkylation and bisANS binding data (Tables 1 and 2 and Figure 3) also show clearly that IKP104, in the presence of colchicine, markedly inhibits the exposure of the sulfhydryl groups and the hydrophobic areas of tubulin, as do vinblastine and other related drugs (*21*−*27*). The most interesting finding is that IKP104 needs colchicine or its related drugs to stabilize tubulin (*3, 4*). These data are very consistent with the earlier observation (*28*), where we found that IKP104-induced tubulin aggregation is significantly reduced in the presence of colchicine.

We decided to see exactly which region of the colchicine binding site is most involved in preventing IKP104 from enhancing tubulin decay. Accordingly, we used the following three compounds: MTPT, which contains the A- and C-rings but not the B-ring; the A-ring analogue TMB; and the C-ring analogue TME. Since the sulfhydryl groups and the hydrophobic areas of tubulin are important reporters about its conformation and function, iodo[^{14}C]acetamide and bisANS were used as probes to study the modulation by the colchicine analogues of the effect of IKP104 on tubulin. From the alkylation data and bisANS binding data (Tables 3 and 4), we found that MTPT has the same inhibitory effect on IKP104 as does colchicine, suggesting that the B-ring binding domain is not involved in terms of the conformational effect of IKP104 on tubulin.

The results with the A- and C-ring analogues were different. The C-ring analogue TME has no effect on IKP104's enhancement of alkylation and has very little effect on its enhancement of bisANS binding (Tables 3 and 5). This suggests that the presence of a C-ring analogue causes little or no alteration in the pattern of the conformational effect of IKP104. In contrast, although the A-ring analogue TMB has no apparent effect on IKP104's enhancement of alkylation, it strongly inhibits its enhancement of bisANS binding (Tables 3 and 5). These two results are actually not contradictory. Since TMB has itself a strong enhancing effect on alkylation, it is quite likely that it is interfering with IKP104's enhancing effect and that the result with the two drugs together is coincidentally the same as that of IKP104 by itself (Table 5). However, TMB by itself has no effect on bisANS binding, so its strong inhibition of IKP104's effect must be due to direct interference with IKP104's interaction with tubulin. Since colchicine does not block the binding of IKP104 at either of its two classes of binding site (Figure 2), it is very unlikely that individual ring analogues such as TMB (or TME) would do so; hence, the most likely way in which TMB can interfere with IKP104's conformational effect is if the TMB binding site (the A-ring binding site) plays a key role in the conformational changes induced in tubulin by IKP104 binding. In contrast, our results suggest that the B- and C-ring binding sites on tubulin play relatively minor roles in mediating IKP104's effect.

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