The Effects of Colchicine Analogues on the Reaction of Tubulin with Iodo\[^{14}\text{C}]\text{acetamide and } N,N'^{-}\text{Ethylenebis(iodoacetamide)}^{*}

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Mary Carmen Roach, Susan Bane§, and Richard F. Ludueña‡

From the Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284 and the 
‡Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

We have previously found (Ludueña, R. F., and Roach, M. C. (1981b) Biochemistry 20, 4444–4450) that colchicine and podophyllotoxin inhibit the alkylation of tubulin by iodo\[^{14}\text{C}]\text{acetamide and the formation of an intrachain cross-link in the } \beta\text{-tubulin subunit by } N,N'^{-}\text{ethylenebis(iodoacetamide)} (EBI). It was not clear whether these effects were due to conformational changes in tubulin induced by drugs or to direct steric blockage of the sulfhydryl groups involved. In an effort to characterize further these phenomena, we have examined the effects of single-ring and bicyclic analogues of colchicine on the reaction of tubulin with iodo\[^{14}\text{C}]\text{acetamide and EBI. We have found that neither the A-ring analogues, 3,4,5-trimethoxybenzyl alcohol, 3,4,5-trimethoxybenzaldehyde, 2,3,4-trimethoxybenzaldehyde, and benzaldehyde, nor the C-ring analogues, tropolone and tropolone methyl ether, inhibited alkylation. Instead, colchicine, podophyllotoxin, and nocodazole, which of the two subunits of tubulin, or about which regions of the tubulin molecule are affected by the drugs. It is not even known to which of the two subunits of tubulin, \(\alpha\) or \(\beta\), these drugs bind.

One approach that has been useful in analyzing the interactions of these drugs with tubulin is the use of analogues of the individual rings of the tricyclic colchicine molecule. Andréau and Timasheff (1982b) have observed that the colchicine-induced stimulation of tubulin’s intrinsic GTPase activity requires the A-ring and is not affected by a C-ring analogue.

We have previously examined the interaction of tubulin with drugs by measuring the effects of drugs on the alkylation of tubulin’s sulfhydryl groups (Ludueña and Roach, 1981a, 1981b). We have reported that colchicine and podophyllotoxin induce a 19–47% reduction in the rate at which these sulfhydryl groups react with iodo\[^{14}\text{C}]\text{acetamide. We have also reported that EBI, a bifunctional analogue of iodoacetamide, makes an intrachain cross-link in the } \beta\text{-subunit of tubulin, causing the subunit to migrate, on discontinuous gels containing } \text{Na dodecyl sulfate, as a faster-moving band designated } \beta^{*}\text{. Ludueña and Roach, 1981a. Colchicine and podophyllotoxin inhibit formation of } \beta^{*}\text{ by 92–94% (Ludueña and Roach, 1981b). From these data, however, we could not conclude whether the effects on alkylation were “steric,” that is due to binding of a drug directly to a region containing the affected sulfhydryls, or “allostERIC,” where the binding of the drug induced a conformational change affecting sulfhydryls located in other regions.}

In order to investigate further the interactions of tubulin with these drugs, we have applied the alkylation method to the approach of using analogues of the A and C rings of

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‡ Present address: Department of Biochemistry, University of Virginia, Charlottesville, VA 22901.

§ To whom correspondence should be addressed.

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\(^{1}\) The abbreviations used are: EBI, \(N,N'^{-}\text{ethylen bis(iodoacetamide)}\); TPMT, 5-(2',3',4'-trimethoxyphenyl)-2-methoxytropane; MES, 2-(N-morpholino)ethanesulfonic acid.

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colchicine. We have found that, whereas colchicine, podophyllotoxin, and nocodazole inhibit the reaction of tubulin with iodo[3H]acetamide neither the A- nor the C-ring analogues do so. Since the bicyclic analogue TMPT, which consists of the A and C rings joined by a single bond, suppresses alkylation, as does combretastatin, which consists of the A ring and a C-like ring joined by a 2-carbon bridge, also suppresses alkylation, it is likely that the suppressive effect of colchicine on alkylation is an "allosteric" one where the majority of affected sulfhydryls is not located at the drug-binding sites.

When we examined the effects of these drugs on β* formation by EBI, we found that the A-ring analogues 3,4,5-trimethoxybenzaldehyde, 2,3,4-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzyl alcohol, and benzaldehyde all inhibit β* formation, as do colchicine, podophyllotoxin, nocodazole, TPMT, and combretastatin. In contrast, the C-ring analogue, tropolone methyl ether, enhanced β* formation. Since all the compounds which inhibited β* formation contained or consisted of analogues of the A ring of colchicine, it appears that the binding of the A ring to tubulin is the determining factor in inhibiting β* formation. Since colchicine and podophyllotoxin have very different effects on the conformation of tubulin, as measured by their effects on alkylation by iodo[3H]acetamide, than do the A-ring analogues, it is conceivable that the effect of the A ring on β* formation is due, not to a conformational change, but to direct steric hindrance of the reaction. In other words, it is possible that one of the sulfhydryls involved in β* formation is located at that site on tubulin where the A ring of colchicine binds.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tropolone, colchicine, iodoacetamide, rabbit muscle aldolase, Na iodoacetate, fast green FCF, conalbumin, and Coomassie Brilliant Blue were from Sigma. Na iodoacetate was further purified by reprecipitation from acetone. Conalbumin was reduced and carboxymethylated by the method of Crestfield et al. (1963). Acrylamide and N,N'-methylenebis(acrylamide) were from the Eastman Kodak Co., Rochester, NY. Acrylamide solutions were routinely filtered and stored as previously described (Ludueña and Roach, 1981a). Vinblastine was from Dr. James Lee, St. Louis University, St. Louis, MO. Combretastatin was the kind gift of Dr. George Pettit, Arizona State University, Tempe, AZ. Microtubule protein was purified from bovine cerebrum, and tubulin was purified from microtubule protein by phosphocellulose chromatography according to the method of Fellous et al. (1977) as previously described (Ludueña and Roach, 1981a). Methanol was omitted from the buffer of Fellous et al. (1977) when tubulin was purified for use in alkylation experiments. All experiments were performed in this buffer.

**Reaction with Iodo[3H]Acetamide**—Tubulin samples were alkylated with iodo[3H]acetamide and then precipitated with trichloroacetic acid and the extent of alkylation of the tubulin determined by the method of Ludueña and Roach (1981c). The binding of [3H] colchicine to tubulin was determined by the filter disc method of Borisy (1972). Protein concentrations were determined by the method of Lowry et al. (1951). Colchicine was dissolved in MES buffer (Fellous et al., 1977) and a sample of nocodazole, TPMT, and the analogues of the A and C rings of colchicine were dissolved in dimethyl sulfoxide immediately prior to use; in experiments where these compounds were used, control samples always contained equivalent concentrations of dimethyl sulfoxide.

**Effect of Colchicine Analogues on the Alkylation of Tubulin by Iodo[3H]Acetamide**—Some of the experiments involving the A-ring analogues of colchicine were done using 3,4,5-trimethoxybenzaldehyde, a compound previously shown by Lin and Hamel (1981) to mimic colchicine’s enhancement of GTPase activity. The analogues 2,3,4-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzyl alcohol, and benzaldehyde were used as well. As expected for colchicine analogues, 3,4,5-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzyl alcohol, and benzaldehyde were used as well. As expected for colchicine analogues, 3,4,5-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzyl alcohol, and benzaldehyde inhibited the binding of [3H]colchicine to tubulin (Table I). All four compounds were capable of enhancing the reaction of tubulin with iodo[3H]acetamide (Table II), although the effect of 3,4,5-trimethoxybenzyl alcohol was significantly smaller than that of the
other two A-ring analogues. That this effect was specific for tubulin was suggested by the fact that 30 mM 3,4,5-trimethoxybenzaldehyde had no effect on the alkylation of aldolase by Iodo[14C]acetamide. In an experiment where triplicate samples of aldolase (1.13 mg/ml) were incubated with 1.36 mM Iodo[14C]acetamide for 60 min at 37 °C, the label incorporated into the aldolase in the presence and absence of 3,4,5-trimethoxybenzaldehyde was 0.64 ± 0.04 and 0.58 ± 0.03 mol/mol, respectively.

It was conceivable that larger increases in alkylation caused by the aldehyde-containing analogues were due in some way to formation of a Schiff base with tubulin and a subsequent conformational change. This would be an effect that need not involve the colchicine-binding site. In order to test this, the effect of acetaldehyde was examined, since this compound would have the aldehyde moiety without any resemblance to colchicine. As can be seen from Table I, a 1-h incubation of tubulin with acetaldehyde had no effect on the binding of [3H]colchicine, whereas all of the A-ring analogues inhibited colchicine binding under the same conditions. After 3 h of incubation, however, acetaldehyde inhibited colchicine binding by 35%, much less than the effect of the A-ring analogues. It is possible that the long-term effect of acetaldehyde was due to Schiff base formation requiring 3 h to show an effect. Nevertheless, it is striking that the A-ring analogues inhibit aldehyde binding after only an hour of incubation, indicating that they are binding to the colchicine site and suggesting that the enhancing effect on alkylation shown by 3,4,5-tri-

| Experiment 1 | Binding of [3H]colchicine to tubulin | Mol [3H]/mol tubulin | % of control |
|--------------|--------------------------------------|----------------------|-------------|
| None         | 0.166 ± 0.008a                       | 100 ± 9%             | 50.88 ± 0.005 | 100 ± 1% |
| 3,4,5-Trimethoxybenzaldehyde, 40 mM | 0.116 ± 0.012 | 70 ± 10 | 0.228 ± 0.003 | 45 ± 1 |
| Acetaldehyde, 40 mM | 0.176 ± 0.012 | 106 ± 12 | 0.328 ± 0.068 | 65 ± 13 |
| Benzyaldehyde, 40 mM | 0.022 ± 0.002 | 13 ± 2 | 0.014 ± 0.003 | 3 ± 1 |

* Aliquots (200 μl) of tubulin (0.15 mg/ml) were incubated in the presence of the indicated compounds and 10 μM [3H]colchicine (204 Ci/mol) for 60 min at 37 °C. All incubations were done in quadruplicate. Colchicine binding to tubulin was then assayed by the filter disc method of Borisy (1972).
* Standard deviation.

| Experiment 2 | Binding of [3H]colchicine to tubulin | Mol [3H]/mol tubulin | % of control |
|--------------|--------------------------------------|----------------------|-------------|
| None         | 0.166 ± 0.008a                       | 100 ± 9%             | 50.88 ± 0.005 | 100 ± 1% |
| 3,4,5-Trimethoxybenzaldehyde, 40 mM | 0.116 ± 0.012 | 70 ± 10 | 0.228 ± 0.003 | 45 ± 1 |
| Acetaldehyde, 40 mM | 0.176 ± 0.012 | 106 ± 12 | 0.328 ± 0.068 | 65 ± 13 |
| Benzyaldehyde, 40 mM | 0.022 ± 0.002 | 13 ± 2 | 0.014 ± 0.003 | 3 ± 1 |

* Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated in triplicate for 60 min at 37 °C in the presence of the indicated compounds. They were then reacted with 1.34 mM Iodo[14C]acetamide (0.49 Ci/mol) for 60 min at 37 °C, and the incorporation of 14C label into each sample was calculated as previously described (Ludueña and Roach, 1981c).
* Standard deviation.
* Aliquots (200 μl) of tubulin (0.15 mg/ml) were incubated in the presence of the indicated compounds and 10 μM [3H]colchicine (204 Ci/mol) for 60 min at 37 °C. All incubations were done in quadruplicate. Colchicine binding to tubulin was then assayed by the filter disc method of Borisy (1972).
* Standard deviation.
* Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated in triplicate for 60 min at 37 °C in the presence of the indicated compounds. They were then reacted with 1.34 mM Iodo[14C]acetamide (0.49 Ci/mol) for 60 min at 37 °C, and the incorporation of 14C label into each sample was calculated as previously described (Ludueña and Roach, 1981c).
Colchicine Analogues and Tubulin Alkylation

Fig. 1. Effect of 3,4,5-trimethoxybenzaldehyde on the alkylation of tubulin by iodo[14C]acetamide. Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated for 60 min at 37 °C for the indicated times with 1.36 mM iodo[14C]acetamide (0.31 Ci/mol) in the presence (O) or absence (C) of 30 mM 3,4,5-trimethoxybenzaldehyde. The radioactivity incorporated into tubulin was measured as described by Ludueña and Roach (1981c). Each time point represents a separate incubation of triplicate samples. Standard deviations are shown except where they are smaller than the symbol.

Fig. 2. Concentration dependence of the effects of colchicine analogues on the alkylation of tubulin by iodo[14C]acetamide. Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated for 60 min at 37 °C in the presence of the indicated concentrations of either TPMT (O), tropolone methyl ether (□), 3,4,5-trimethoxybenzaldehyde (△), or 3,4,5-trimethoxybenzaldehyde with 20 μM vinblastine (▲). One set of aliquots contained 20 mM tropolone methyl ether and 15 mM 3,4,5-trimethoxybenzaldehyde (■). After the incubation with the analogues, the samples were incubated for 60 min at 37 °C with 1.34 mM iodo[14C]acetamide (0.49 Ci/mol). Incorporation of label into tubulin was measured as in Fig. 1 and is expressed as a percentage of the control value obtained in the absence of the colchicine analogue. The figure is a composite of the results obtained in 4 separate experiments whose control values were as follows: Experiment 1 (△), 3.37 ± 0.02 mol 14C/mole tubulin; Experiment 2 (□, ■), 4.37 ± 0.10 mol 14C/mole tubulin; Experiment 3 (○), 4.81 ± 0.11 mol 14C/mole tubulin; Experiment 4 (▲), 5.05 ± 0.03 mol 14C/mole tubulin in the presence and 1.04 ± 0.05 mol 14C/mole tubulin in the absence of 20 μM vinblastine. Each point represents the average of two or three incubations. Standard deviations are shown.

mide for 60 min at 37 °C, the extent of incorporation of label into aldolase in the presence and absence of tropolone was 0.49 ± 0.09 and 0.48 ± 0.03 mol/mol.

Tropolone methyl ether caused a more moderate enhancement of alkylation although the effects were only visible at concentrations of 10 mM or higher (Fig. 2). At this concentration range, tropolone methyl ether inhibited colchicine binding (Table I). The effect of tropolone methyl ether was apparently due to an increase in the rate of alkylation of tubulin by iodo[14C]acetamide (not shown). When tropolone methyl ether and 3,4,5-trimethoxybenzaldehyde were tested in combination, their effects on incorporation of iodo[14C]acetamide were indistinguishable from those of 3,4,5-trimethoxybenzaldehyde by itself (Fig. 2).

In order to see what effect linking the A and C rings would have on alkylation, experiments were done with TPMT and combretastatin. TPMT was able to inhibit the alkylation of tubulin by iodo[14C]acetamide (Fig. 2) at concentrations where it also inhibited binding of [3H]colchicine to tubulin (Table I). Half-maximal inhibition was obtained at about 2–10 μM TPMT. The inhibition of alkylation was due to a decrease in the rate of reaction of tubulin with iodo[14C]acetamide (not shown). As can be seen in Table II, combretastatin also inhibits alkylation. This bicyclic compound is an analogue of colchicine lacking the acetamide group on the B ring and the covalent bond joining the A and C rings; combretastatin also has colchicine’s C ring replaced by a phenylene group with methoxy and hydroxyl groups at positions equivalent, respectively, to those where a methoxy and ketone group occur on the C ring of colchicine (Pettit et al., 1982; Hamel and Lin, 1985). Combretastatin thus consists of the A ring and a C-like ring joined by a 2-carbon bridge and, in terms of the B ring, is complementary to TPMT. This inhibition of alkylation is in marked contrast to the effects of the individual A- and C-ring analogues, which enhance alkylation. Even when 3,4,5-trimethoxybenzaldehyde and tropolone methyl ether were tested together, they failed to inhibit alkylation (Fig. 2), suggesting that a bond or bridge, joining the A and C rings, is critical in determining the effect of the compound on the alkylation of tubulin by iodo[14C]acetamide.

The Effect of Colchicine Analogues on β* Formation by EBI—Fig. 3 shows that the rate of formation of β* by EBI is strongly inhibited by 3,4,5-trimethoxybenzaldehyde. However, as shown above, the effect of single ring analogues of colchicine on the alkylation of tubulin by iodo[14C]acetamide is to enhance it. We have previously noted that drugs which inhibit alkylation also inhibit formation of a nonspecific high

Fig. 3. Effect of colchicine analogues on the rate of formation of β* (A) and the rate of disappearance of β (B) in tubulin reacted with EBI. Aliquots (250 μl) of tubulin (0.66 mg/ml) containing reduced and carboxymethylated conalbumin (0.2 mg/ml) were incubated for the indicated lengths of time at 30 °C with 0.91 mM EBI and in the absence (O) or presence of either 20 mM tropolone methyl ether (□), 100 μM TPMT (○), 10 mM 3,4,5-trimethoxybenzaldehyde (△), or 10 mM 3,4,5-trimethoxybenzaldehyde and 20 μM vinblastine (▲). The samples were processed as described under "Experimental Procedures." The figure shows the % β* (A) and the % βres (B) in each sample. Each point represents a separate incubation.
molecular weight cross-linked aggregate by EBI, regardless of their effects on β* formation (Ludueña and Roach, 1981b). As one might expect, analogues which strongly enhanced alkylation also enhanced formation of this aggregate, which made the decreased yield of β* difficult to interpret. For example, in the presence of EBI and 30 mM 3,4,5-trimethoxybenzaldehyde about 88% of α, 82% of β*, and 74% of β2 were incorporated into this aggregate. It was, therefore, not clear if the analogue inhibited β* formation by directly blocking that particular reaction or by enhancing a competing reaction. Fig. 3, however, suggests that the former may be the case, since 3,4,5-trimethoxybenzaldehyde slowed down the rate of disappearance of β*.

In order to minimize the contribution of nonspecific cross-linking, a similar experiment was done in the presence of vinblastine, a drug known to inhibit nonspecific cross-linking by EBI, while enhancing formation of β* (Ludueña and Roach, 1981b). When the effect of 3,4,5-trimethoxybenzaldehyde on β* formation by EBI in the presence of vinblastine was examined (Figs. 3–5), it was found that the yield of β* was suppressed by 80% at a concentration of 15 mM 3,4,5-trimethoxybenzaldehyde. Even at concentrations below 15 mM, 3,4,5-trimethoxybenzaldehyde significantly inhibited β* formation, half-maximal inhibition being obtained with 2 mM 3,4,5-trimethoxybenzaldehyde. Most important, as the concentration of 3,4,5-trimethoxybenzaldehyde increased from 0 to 15 mM, the yield of residual (non-cross-linked) β* increased from 22 to 47% and that of β* decreased from 58 to 11% (Fig. 5), indicating that 3,4,5-trimethoxybenzaldehyde was actually inhibiting formation of the β* cross-link, rather than merely increasing the transformation of β* into a competing structure such as the cross-linked aggregate.

As shown in Fig. 3, in the presence of vinblastine, 3,4,5-trimethoxybenzaldehyde inhibited the rate of formation of β* and decreased the rate of disappearance of β*, consistent with what was observed in the absence of vinblastine.

The effects of 3,4,5-trimethoxybenzyl alcohol and benzaldehyde on the reaction of EBI with tubulin were also examined (Table III). As can be seen, these analogues significantly inhibiting β* formation by EBI while concomitantly increasing the yield of βres. Since 3,4,5-trimethoxybenzyl alcohol and benzaldehyde had little effect on nonspecific cross-linking, as represented by βagg, it is likely that the decreased yield of β* was due directly to inhibition of cross-link formation.

Interestingly, acetaldehyde inhibited β* formation and en-

**Table III**

| Addition | Yield of β* derivative (% of total β*) |
|----------|---------------------------------------|
|          | β* | βres | βagg |
| Experiment 1* | | | |
| None | 48 ± 3 | 32 ± 1 | 20 ± 4 |
| 3,4,5-T trimethoxybenzaldehyde, 60 mM | 33 ± 3 | 45 ± 4 | 22 ± 7 |
| Vinblastine, 40 μM | 64 ± 3 | 28 ± 0.2 | 8 ± 3 |
| Vinblastine, 40 μM, and 3,4,5-trimethoxybenzaldehyde, 60 mM | 40 ± 1 | 46 ± 2 | 14 ± 1 |
| Experiment 2 | | | |
| None | 57 ± 4 | 25 ± 1 | 18 ± 5 |
| 3,4,5-T trimethoxybenzaldehyde, 8 mM | 19 ± 1 | 54 ± 4 | 27 ± 5 |
| 3,4,5-T trimethoxybenzaldehyde, 40 mM | 42 ± 4 | 35 ± 1 | 23 ± 4 |
| Acetaldehyde, 8 mM | 54 ± 6 | 28 ± 3 | 19 ± 8 |
| Benzaldehyde, 8 mM | 47 ± 4 | 43 ± 4 | 10 ± 8 |
| Benzaldehyde, 40 mM | 15 ± 1 | 55 ± 4 | 30 ± 4 |
| Experiment 3* | | | |
| None | 65 ± 4 | 24 ± 1 | 11 ± 5 |
| Acetaldehyde, 40 mM | 32 ± 2 | 39 ± 2 | 22 ± 4 |
| Experiment 4* | | | |
| None | 59 ± 2 | 25 ± 1 | 16 ± 2 |
| Combretastatin, 50 μM | 10 ± 1 | 88 ± 1 | 3 ± 1 |

* Aliquots (250 μl) of tubulin (0.66 mg/ml) containing reduced and carbamylated conalbumin (0.2 mg/ml) were incubated for 60 min at 37 °C in the presence of 0.9 mM EBI and of the indicated drugs. Samples were incubated in triplicate. The yields of β*, βres, and βagg were measured as described under “Experimental Procedures.”

* Standard deviation.

* The experimental conditions were identical to those used in Experiment 1, except that all samples contained 20 μM vinblastine and the incubation was at 28 °C.

* Experimental conditions were identical to those used in Experiment 1, except that all incubations were in quadruplicate and all samples contained 20 μM vinblastine.

**Fig. 4.** Gel electrophoretic analysis of samples of tubulin alkylated with EBI in the presence of colchicine analogues. Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated with drugs and analogues for 60 min at 37 °C and then with 0.89 mM EBI (samples 2, 3, and 5-11) or without EBI (samples 1 and 12) for 60 min at 30 °C. Sample 4 was not preincubated at 37 °C, and the concentration of EBI that was used was 0.91 mM. Samples were then reduced and carboxymethylated and analyzed on a 8% polyacrylamide gel in the system of Laemmli (1970). Samples were incubated as follows: 1, no drug, no EBI; 2, no drug; 3, colchicine, 10 μM; 4, podophyllotoxin, 20 μM; 5, nocodazole, 20 μM; 6, TPMT, 200 μM; 7, tropolone methyl ether, 20 mM; 8, tropolone methyl ether, 42 mM, and vinblastine, 20 μM; 9, 3,4,5-trimethoxybenzaldehyde, 15 mM; and vinblastine, 20 μM; 11, vinblastine, 20 μM; 12, no EBI. The α, β*, β2, and β* bands are designated. The gel was stained in Coomassie Blue. Electrophoresis was from top to bottom.
hanced the yield of \( \beta_{\text{res}} \), suggesting that it was acting as a genuine inhibitor of the formation of the cross-link.

As shown in Table II, tropolone significantly enhanced alkylation of tubulin by iodo\[^{14}\text{C}]\text{acetamide. Tropolone also enhanced nonspecific cross-linking by EBI to a very large extent. For example, in the presence of 40 mM tropolone, 97% of \( \alpha \), 92% of \( \beta_2 \), and 83% of \( \beta_1 \) disappeared from the gel. Even the addition of vinblastine did not significantly diminish this effect of tropolone. In one experiment, for instance, in the presence of 20 \( \mu \text{M} \) vinblastine, 20 mM tropolone caused the disappearance from the gel of 63% of \( \alpha \), 54% of \( \beta_2 \), and 65% of \( \beta_1 \). In contrast to the A-ring analogues, however, tropolone did not induce as great a decrease in the yield of \( \beta^* \) relative to \( \beta_1 \) as did the A-ring analogues. For example, 20 mM tropolone generated \( \beta^*/\beta_1 \) ratios of 1.15 and 0.93 in the presence and absence, respectively, of 20 mM vinblastine. Nevertheless, the strong enhancement of nonspecific cross-linking by tropolone made this compound an unsatisfactory choice for examining the effect of C-ring analogues on \( \beta^* \) formation.

Tropolone methyl ether caused only a small increase in nonspecific cross-linking (Fig. 6). Interestingly, tropolone methyl ether did not significantly diminish the yield of \( \beta^* \), even at concentrations where it enhanced alkylation by iodo\[^{14}\text{C}]\text{acetamide. In fact, tropolone methyl ether actually increased the yield of \( \beta^* \) relative to \( \beta_1 \) (Figs. 3, 4, and 6). For example, when the tropolone methyl ether concentration increased from 0 to 20 mM, the yield of residual \( \beta_1 \) decreased by 17% while the yields of \( \beta^* \) and aggregated \( \beta_1 \) increased by 9 and 8%, respectively (Fig. 6). In a similar experiment done in the presence of 20 \( \mu \text{M} \) vinblastine, an increase of the tropolone methyl ether concentration from 0 to 20 mM caused a 9% decrease in the yield of residual \( \beta_1 \), while the yields of \( \beta^* \) and aggregated \( \beta_1 \) increased by 7 and 3%, respectively. These results suggest that the increased yield of \( \beta^* \) relative to \( \beta_1 \) was caused by tropolone methyl ether due at least in part to the direct enhancement of formation of the cross-link which generates \( \beta^* \) as well as by enhancement of nonspecific cross-linking. Only at high concentrations (40 mM) did tropolone methyl ether induce a decrease in the yield of \( \beta^* \), this being accompanied by a large increase in the yield of cross-linked aggregated \( \beta_1 \) (Fig. 6). When tropolone methyl ether and 3,4,5-trimethoxybenzaldehyde were tested together, the effect on \( \beta^* \) formation was the same as that of 3,4,5-trimethoxybenzaldehyde alone (Fig. 6). The enhancing effect of tropolone methyl ether on \( \beta^* \) formation appeared to have a complex time dependence, being less pronounced at longer reaction times. However, tropolone methyl ether strongly enhanced the rate of disappearance of \( \beta_1 \), suggesting that at longer reaction times (after 30 min), tropolone methyl ether enhanced the formation of aggregated cross-linked \( \beta_1 \) at the expense of \( \beta^* \) (Fig. 2).

When tested in the presence of EBI, TPMT markedly decreased \( \beta^* \) formation (Fig. 7). The effect was half-maximal at a TPMT concentration of about 8 \( \mu \text{M} \). Like colchicine (Ludueña and Roach, 1981b), TPMT also induced a decrease in nonspecific cross-linking. The suppression of \( \beta^* \) formation by TPMT appeared to be a simple inhibition of the reaction rate (Fig. 3) and was accompanied by a strong inhibition of the rate of disappearance of \( \beta_1 \). In similar fashion, combretastatin inhibition both the formation of \( \beta^* \) and the disappearance of \( \beta_1 \) (Table III).

**DISCUSSION**

**Effect of Colchicine Analogues on the Alkylation of Tubulin by Iodo\[^{14}\text{C}]\text{acetamide}**—It is clear from the results presented in Table II and Figs. 1 and 2 that the analogues of the individual A and C rings of colchicine either enhance or at least do not inhibit the alkylation of tubulin by iodo\[^{14}\text{C}]\text{acetamide at concentrations where they are binding specifically to tubulin as shown by their inhibition of colchicine binding} (Table I). This result is in marked contrast to the effects of colchicine, podophytoxin, nocodazole, TPMT, and combretastatin, all of which inhibit the reaction of tubulin
with iodido\(^{125}\)Iacetamide. It may be argued that 3,4,5-trimethoxybenzaldehyde, 2,3,4-trimethoxybenzaldehyde, and benzaldehyde could react covalently with tubulin at some site distinct from the colchicine-binding site to form a Schiff base and that such a reaction could affect the conformation of the tubulin molecule by a unique mechanism that would not involve the colchicine-binding site. By this argument the strong enhancement of alkylation caused by these A-ring analogues could conceivably be a result of such a conformational change. However, acetaldehyde, which has an aldehyde moiety but no resemblance to colchicine, has only a small effect on alkylation (Table I). On the other hand, the A-ring analogue, 3,4,5-trimethoxybenzyl alcohol, which lacks the aldehyde moiety, also has little effect on alkylation. It is conceivable that the combination of the resemblance to colchicine and the presence of the aldehyde moiety may be responsible for the strong enhancement of alkylation, perhaps as a result of binding at the colchicine site followed by formation of a Schiff base with a neighboring favorably oriented amino group. It is interesting that much stronger inhibition of colchicine binding by the A-ring analogues was observed with 3,4,5-trimethoxybenzaldehyde and benzaldehyde than with 3,4,5-trimethoxybenzyl alcohol or acetaldehyde. In fact, acetaldehyde only became inhibitory after a long incubation (Table I). The important point, however, is not to what extent the A- and C-ring analogues enhance alkylation of tubulin by iodido\(^{125}\)Iacetamide but that they do not inhibit it, in contrast to colchicine and the bifunctional analogues which do inhibit it.

It is interesting that TPMT suppresses alkylation because structurally TPMT consists of the A and C rings joined by a covalent bond. The fact that the combination of 3,4,5-trimethoxybenzaldehyde and tropolone methyl ether also enhances alkylation (Fig. 2) suggests that the fact of the A and C rings being joined, as they are in TPMT, combretastatin, and colchicine, is all important in determining whether alkylation will be inhibited or enhanced. If the inhibition of alkylation by colchicine and TPMT is purely a steric effect, that is due to direct blockage of the reacting sulfhydryls, then the sulfhydryls in question would have to be located precisely in that portion of the binding site which is closest to the bond joining the A and C rings. Although the precise number of sulfhydryls affected by colchicine has not been determined, Ludueña and Roach (1981b) have shown that these sulfhydryls are located on both the \(\alpha\) and \(\beta\) subunits. In other words, for the steric model to be true, the bond joining the A and C rings would not only have to be located close to the affected sulfhydryl, it would also have to straddle the \(\alpha/\beta\) interface. The fact that combretastatin inhibits alkylation makes this model less likely, however, because the bond which joins the A and C rings in TPMT is absent in combretastatin. Instead the A ring and the ring equivalent to the C ring are joined by a 2-carbon bridge similar to the outer part of colchicine’s B ring. It is difficult to explain by a simple steric model how single A- and C-ring analogues, even in combination, fail to suppress alkylation while any compound in which the two rings are connected, no matter how, will suppress alkylation.

The results presented in this paper lend themselves to another model, an allosteric one, where colchicine binding to tubulin induces a conformational change in the molecule which affects several sulfhydryl groups on both subunits. The conformational change would be dependent upon the presence of the bond joining the A and C rings. By this model, the single ring analogues of colchicine would either induce conformational changes in tubulin or destabilize its conformation in such a way as to enhance the alkylation of tubulin. Such a model is in apparent contradiction to the results of Andreu and Timasheff (1982b), who find that tropolone methyl ether and colchicine affect the circular dichroism spectrum of tubulin in similar fashions, while podophyllotoxin, considered here as an A-ring analogue, has a different effect. It must be considered, however, that a slight shift in the relative positions of two domains in tubulin induced by the binding of a ligand could conceivably have little or no effect on the circular dichroism spectrum of the molecule but could greatly alter the accessibility of certain sulfhydryl groups to alkylating agents. Along the same lines, Andreu and Timasheff (1982a) find that the observed free energies of tubulin binding to colchicine, tropolone methyl ether, and the A-ring analogue N-acetylneuraminic acid is consistent with a model whereby the bindings of the A- and C-ring moieties of colchicine to tubulin are not altered by the existence of the covalent bond joining the two rings. Our results, in contrast, would imply that the existence of this bond causes changes in the accessibility of certain sulfhydryl groups but that the free energy of such a change could be small enough to be within experimental error of the free energies estimated by Andreu and Timasheff (1982a). It is probable, therefore, that the conformational change which we postulate to account for colchicine’s inhibition of alkylation is just a small part of the larger conformational change which Andreu and Timasheff (1982a) propose in which the binding of the C-ring moiety of colchicine facilitates the binding of the A-ring moiety.

It is interesting that Andreu et al. (1984) have recently shown that TPMT affects tubulin’s intrinsic fluorescence, circular dichroism, and GTPase activity in a manner similar to that of colchicine. They propose that the conformation of the tubulin molecule when it is bound to TPMT is similar to its conformation when it is bound to colchicine. This is consistent with our observation that TPMT and colchicine both inhibit the alkylation of tubulin by iodido\(^{125}\)Iacetamide. As shown in Table II, podophyllotoxin and nocodazole also inhibit alkylation by iodido\(^{125}\)Iacetamide. It is possible that these effects are also due to putative conformational changes induced by these drugs, but the possibility cannot be eliminated that there is a steric inhibition of alkylation due to those portions of podophyllotoxin and nocodazole which bind to areas on the tubulin molecule where colchicine does not bind. In order to investigate this possibility it would be necessary to examine the effects of analogues of individual portions of these drugs, such as the tetracyclic component of podophyllotoxin.

The Effect of Colchicine Analogues on the Formation of the \(\beta^*\) Cross-link by EBI—Previously published work (Ludueña and Roach, 1981b) and the data shown here indicate that colchicine, podophyllotoxin, nocodazole, TPMT, and combretastatin are potent inhibitors of \(\beta^*\) formation by EBI, suggesting that these compounds, when they bind to tubulin, have a strong effect on at least one of the sulfhydryl groups involved in \(\beta^*\) formation. When the effects of the analogues of the individual colchicine rings are examined, it is seen that only the A-ring analogues can suppress \(\beta^*\) formation. Of the two C-ring analogues used in this study, tropolone induced too much nonspecific cross-linking to allow accurate estimation of a direct effect on \(\beta^*\) formation, while the other, tropolone methyl ether, directly enhanced formation of \(\beta^*\). In contrast, the A-ring analogues 3,4,5-trimethoxybenzaldehyde, 2,3,4-trimethoxybenzaldehyde, 3,4,5-trimethoxybenzyl...
alcohol, and benzaldehyde suppressed $\beta^*$ formation. In the absence of vinblastine, the A-ring analogues caused a large increase in the formation of cross-linked aggregate by EBI, an effect which did not allow demonstration of whether $\beta^*$ formation was decreased by true inhibition of cross-linking or by formation of a competing cross-linked product. However, in the presence of vinblastine, which greatly diminishes nonspecific alkylation (Ludueña and Roach, 1981b) the A-ring analogues induced a lowered yield of $\beta^*$ and a higher yield of residual (noncross-linked) $\beta$. Apparently, therefore, these compounds also act by specifically inhibiting the reaction with EBI of at least one of the sulfhydryl groups involved in $\beta^*$ formation. It thus seems that the ability to influence these particular sulfhydryl groups is restricted to those compounds which contain a trimethoxyphenyl ring or, in the case of nocodazole, an equivalently placed phenylene moiety. As shown above, colchicine, podophyllotoxin, nocodazole, TPMT, and combretastatin are potent inhibitors of the alkylation of tubulin by iodo-[14C]acetamide. The evidence suggests, particularly in the case of colchicine, that this effect is conformational in nature, that is the affected sulfhydryls are not likely to be located at the drug-binding site. In contrast, the A-ring analogues enhance alkylation of sulfhydryl groups which again must not be located at their binding site. In addition, Andreu and Timasheff (1982b) have shown that podophyllotoxin and colchicine induce different conformational effects on the tubulin molecule. The fact that all of these compounds, whose conformational effects on the tubulin molecule appear to vary considerably, all affect the same sulfhydryl or sulfhydryls the same way, suggests that this latter effect is not due to a conformational change induced by the ligand but to direct hindrance of the sulfhydryl or sulfhydryl by the ligand. In other words, it is likely that these sulfhydryls are located at or near to the region on the tubulin molecule where the A ring of colchicine binds, this presumably being the place where the binding sites of podophyllotoxin and nocodazole overlap colchicine’s (Cortese et al., 1977; Lin and Hamel, 1981). It is conceivable, however, that even though the overall conformational effects on tubulin of different ligands may be very different, there may be a specific region of tubulin where these effects are the same, and it is certainly possible that one or both of the sulfhydryl groups involved in $\beta^*$ formation may be located in this region. Another complicating factor is that whereas $\beta^*$ formation requires two sulfhydryls, inhibition of $\beta^*$ formation requires that only one sulfhydryl be prevented from reacting with EBI. It is conceivable, though unlikely, that the different ligands act by suppressing the reactivity of different sulfhydryls. If this were the case, then our argument based on a common mechanism of action of different ligands would be less convincing and the likelihood of the effects being allosteric rather than steric would be higher.

It is interesting that acetaldehyde should also suppress $\beta^*$ formation (Table III), since this compound does not bind at the colchicine-binding site (Table I). This result raises the possibility that inhibition of $\beta^*$ formation is due to a reaction of an aldehyde group with one of the reactive sulfhydryls, which need not involve the colchicine-binding site. However, the fact that 3,4,5-trimethoxybenzyl alcohol is a good inhibitor of $\beta^*$ formation (Table III) suggests that the presence of the aldehyde group is not necessary for this to happen. Conceivably, acetaldehyde forms a Schiff base elsewhere in the tubulin molecule which coincidentally prevents $\beta^*$ formation in a different way, perhaps by being located between the two $\beta^*$ sulfhydryls.

The tentative model advanced here, that the sulfhydryls involved in $\beta^*$ formation are located at the site where the A ring of colchicine binds, has three interesting corollaries. The first and most obvious is that these sulfhydryls are on the $\beta$ subunit of tubulin and that the colchicine-binding site is located, at least in part, on $\beta$. This is an apparent contradiction to the preliminary results of Barnes et al. (1982) who find that a photoaffinity analogue of colchicine can be covalently linked to the $\alpha$ chain. However, this analogue has its photoactive portion attached to the B ring and the fact that it cross-links to $\alpha$ may simply mean that the B ring is no further away from the $\alpha$ chain than the length of the photoactive moiety. Such a conclusion is in no way contradictory to a model where the A ring binds to $\beta$. It is also possible that the colchicine-binding site overlaps both the $\alpha$ and $\beta$ subunits.

A second corollary of the model derives from the observations by Palanivelu and Ludueña (1982) and Ludueña et al. (1982) that one or both of the sulfhydryls involved in $\beta^*$ formation is critical for microtubule assembly and that a molecule of tubulin that has reacted with EBI to form $\beta^*$ cannot assemble. If the model advanced here is correct and one or both of these sulfhydryls are located at the site where colchicine, podophyllotoxin, and nocodazole bind, then we may deduce that this region of the tubulin molecule must have a critical role in determining whether a molecule of tubulin may polymerize. Polymerization to microtubules would be inhibited either by a reagent which reacts with the sulfhydryl at this site or by a drug, such as colchicine, which covers them. Whether this site serves as a tubulin-tubulin binding site in microtubule assembly or whether the events at this site affect a more distant tubulin-tubulin interaction site is unclear.

A third corollary of this model derives from the observation in Figs. 6 and 8 that tropolone methyl ether enhances $\beta^*$ formation. If the sulfhydryl groups involved in $\beta^*$ formation are located where the A ring binds, then it is probable that the binding of tropolone methyl ether to tubulin causes a conformational change which affects the A-ring-binding site, perhaps moving it so that the orientation of the sulfhydryl groups is altered in such a way as to facilitate $\beta^*$ formation. This is very similar to what Andreu and Timasheff (1982a) have proposed, namely that the $\alpha$ chain of colchicine binds first and that it induces a conformational change which affects the A-ring-binding site. It is likely, but not definite, that these two sites are one and the same. Experiments, currently in progress, to locate the $\alpha$-ring-binding site and to identify the sulfhydryl groups which have their alkylation affected by the binding of colchicine should greatly increase our understanding of the mechanisms of action of colchicine and other anti-mitotic drugs.

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