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Biochemistry

2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site

(angiogenesis inhibition/glutamate/diethylstilbestrol/combretastatin A-4/17-ethynylestradiol)

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ABSTRACT **A metabolite of estradiol, 2-methoxyestradiol (2ME), inhibits angiogenesis in the chicken embryo chorioallantoic membrane assay. Since 2ME causes mitotic perturbations, we examined its interactions with tubulin. In our standard 1.0 M glutamate system (plus 1.0 mM $MgCl_2$ at 37°C), superstoichiometric concentrations (relative to tubulin) of 2ME inhibited the nucleation and propagation phases of tubulin assembly but did not affect the reaction extent. Although polymer formed in the presence of 2ME was more cold-stable than control polymer, morphology was little changed. Under suboptimal reaction conditions (0.8 M glutamate/no $MgCl_2$ at 26°C), substoichiometric 2ME totally inhibited polymerization. No other estrogenic compound was as effective as 2ME as an inhibitor of polymerization or of the binding of colchicine to tubulin. Inhibition of colchicine binding was competitive (K_i, 22 μM). Thus, a mammalian metabolite of estradiol binds to the colchicine site of tubulin and, depending on reaction conditions, either inhibits assembly or seems to be incorporated into a polymer with altered stability properties.**

There is growing evidence that estrogenic compounds affect cell division and act directly on microtubules by interacting with tubulin. The most extensively studied of these compounds is the synthetic analog diethylstilbestrol (DES; Fig. 1, compound 1) and related agents (1–3). These drugs cause disturbances in mitosis, inhibit microtubule assembly at relatively high concentrations, and inhibit the binding of colchicine to tubulin. Estradiol (compound 2), the major estrogenic hormone of human beings, also causes disturbances of mitosis in cultured cells (1, 4, 5). These perturbations include aneuploidy, multinucleation, and mitotic arrest, and estradiol has been reported to inhibit the polymerization of rat brain tubulin (6). Seegers *et al.* (5) found that 2-methoxyestradiol (2ME; compound 3), a metabolite of both estradiol (7) and the oral contraceptive agent 17-ethynylestradiol (compound 4), was more potent than estradiol in producing mitotic perturbations and proposed that it was 2ME rather than estradiol that caused the observed disturbances. Although 2-methoxyestrogens are extremely weak in binding to cytosol estrogen receptors (8), 2ME is found in blood and urine after sequential hepatic hydroxylation and methylation of estradiol (7).

We recently found that 2ME inhibited angiogenesis in the chicken embryo chorioallantoic membrane assay of Crum *et al.* (9). Disks of 2ME (100 μg) produced large avascular zones 48 hr after implantation on a 6-day embryo, similar to *in vitro* results of others (10). In attempting to define the mechanistic basis, we observed that 2ME inhibited microtubule assembly. This finding led us to study the interactions of 2ME with purified tubulin.

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MATERIALS AND METHODS

Materials. Purified bovine brain tubulin and H_2-CSA4 were prepared as described (11, 12). Monosodium glutamate (2.0 M) was adjusted to pH 6.6 with HCl. 2ME, 2-fluoroestradiol, 2-methoxy-17-ethynylestradiol, 2-methoxyestradiol 3-*O*-methyl ether, 2-bromoestradiol, and 4-methoxyestradiol were obtained from Steraloids (Wilton, NH); podophyllotoxin was from Aldrich; colchicine, DES, estradiol, estrone, estriol, 17-ethynylestradiol, and estradiol 3-*O*-methyl ether were from Sigma; 2-methoxyestriol and 4-methoxyestradiol 3-*O*-methyl ether were from Research Plus (Bayonne, NJ); and [^{3}H]colchicine was from DuPont. CSA4 was a gift of G. R. Pettit (Arizona State University).

Methods. Tubulin polymerization was followed in Gilford recording spectrophotometers equipped with electronic temperature controllers. A drug–tubulin preincubation (0.24-ml reaction mixture) without GTP was performed for 15 min at 26 or 37°C, as indicated. The samples were chilled on ice, and GTP was added in 10 μl. Further details are provided for individual experiments. Reaction mixtures were transferred to cuvettes held at 0°C, baselines were established, and polymerization was initiated by setting the temperature controller to the desired temperature. Turbidity development was followed at 350 nm. The IC_{50} value was defined as the drug concentration required to inhibit extent of assembly 50% after a 20-min incubation. IC_{50} values were determined graphically (linear scale) in at least three experiments.

The binding of [^{3}H]colchicine to tubulin was measured on DEAE-cellulose filters (12). Reaction mixtures contained 1.0 M monosodium glutamate (pH 6.6), 0.1 M glucose 1-phosphate, albumin (0.5 mg/ml), 1.0 mM $MgCl_2$, 1.0 mM GTP, and tubulin, [^{3}H]colchicine, and inhibitors as indicated.

RESULTS

With microtubule protein and with tubulin plus microtubule-associated proteins, weak inhibition of assembly occurred with 2ME ($IC_{50} > 20$ μM). Because estramustine phosphate (Fig. 1, compound 5) interacts with microtubule-associated proteins (13, 14), we wanted to determine which microtubule component interacted with 2ME.

We used the glutamate-induced polymerization of purified tubulin, beginning with our standard reaction condition (12) with 10 μM tubulin. In the experiment of Fig. 2*A*, three high concentrations of 2ME (20, 40, and 75 μM; curves 2–4, respectively) were compared to a control (curve 1). Although there was a progressive delay in the onset of polymerization, reaction plateau values were similar under all conditions. We monitor cold-induced depolymerization in experiments with potential inhibitors of microtubule assembly. We observed progressive resistance to cold as the 2ME concentration

Abbreviations: DES, diethylstilbestrol; 2ME, 2-methoxyestradiol; CSA4, combretastatin A-4; H_2-CSA4, dihydrocombretastatin A-4.

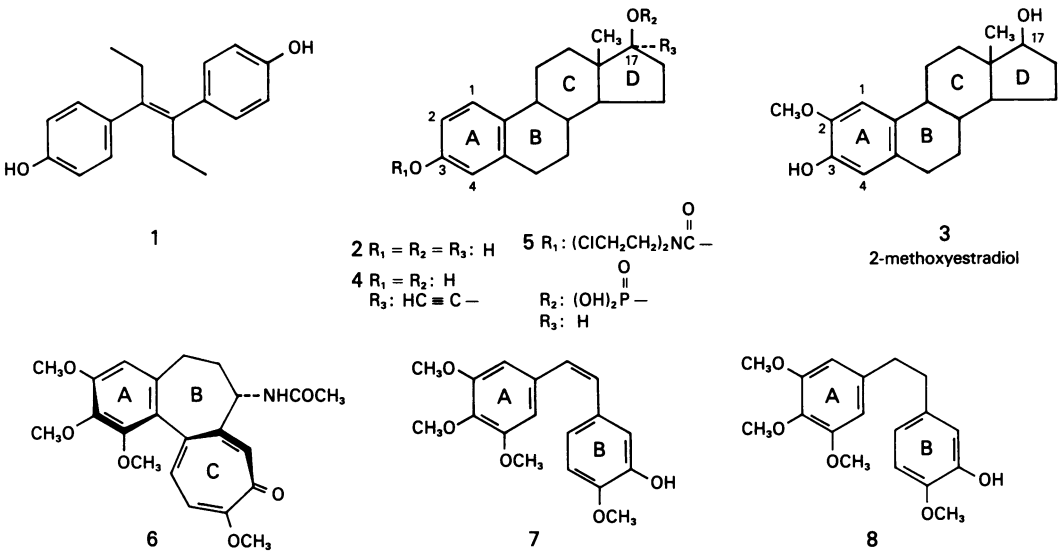

FIG. 1. Estrogenic compounds, colchicine, (**6**) combretastatin A-4 (**7**; CSA4), and dihydrocombretastatin A-4 (**8**; H_2-CSA4).

increased, which could be enhanced by performing the depolymerization in two stages, as in Fig. 2*A*, with the temperature of the reaction first reduced to 10°C and then to 0°C. Polymerization in the presence of 2ME required GTP.

These observations demonstrated major perturbation of the glutamate-induced polymerization reaction by 2ME, leading us to examine polymer formed with and without drug with the electron microscope (Fig. 3). The glutamate polymer formed in the absence of drug (15) consisted largely of open ribbons of protofilaments (Fig. 3 *A* and *B*). Although we had anticipated polymer with altered morphology, perhaps twisted ribbons (16), there was no major change in morphology (Fig. 3 *D* and *E*). The ribbons were narrower in the presence of the steroid, and a significant number appeared to have the lucent walls observed in microtubules. To determine whether 2ME shifted the glutamate polymer from ribbons to microtubules, thin sections were examined with the electron microscope. Areas with a relatively large number of cross-sectioned polymers are shown in Fig. 3 *C* (no drug) and *F* (+2ME). There was some increase in tubular structures in the presence of the estrogen derivative, but open ribbons remain the predominant polymer.

We previously observed changes in polymer stability, reduced polymerization rates, and unaltered plateau readings with a number of colchicine-site antimitotic agents.‡ We minimized what seemed to be drug-dependent aberrant polymerization reactions by reducing the reaction temperature and the $MgCl_2$ and glutamate concentrations (12). This permitted ready determination of IC_{50} values for these drugs, for progressive inhibition of assembly replaced more complex patterns similar to that shown in Fig. 2*A*. These suboptimal conditions also yielded significantly lower IC_{50} values than were obtained with the standard condition for agents that could be examined under both conditions (12).

Such a suboptimal condition was useful with 2ME. Fig. 2*B* demonstrates progressive inhibition of polymerization of 12 μM tubulin with 1–4 μM drug. Unlike the superstoichiometric concentrations (relative to tubulin) required to observe the effects shown in Fig. 2*A*, substoichiometric 2ME completely inhibited polymerization in the experiment of Fig. 2*B*.

Table 1 summarizes IC_{50} values obtained in the suboptimal condition for 2ME, estradiol, 17-ethynylestradiol and its 2-methoxy derivative, and a number of related compounds. For comparison, we determined IC_{50} values in this system for colchicine (Fig. 1, compound **6**), podophyllotoxin, DES,

‡Up to 100 μM colchicine does not induce such aberrant polymerization reactions in 1.0 M glutamate under any reaction condition yet examined.

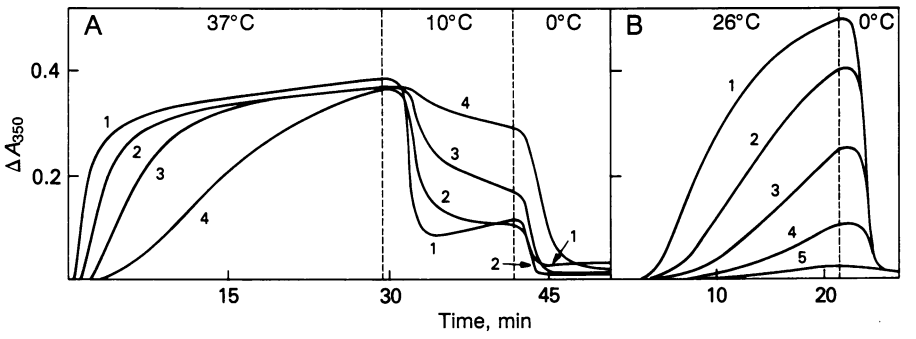

FIG. 2. Effects of 2ME on glutamate-induced tubulin polymerization. (*A*) Reaction mixtures contained 1.0 M monosodium glutamate (pH 6.6), tubulin at 1.0 mg/ml, 1.0 mM $MgCl_2$, 4% (vol/vol) dimethyl sulfoxide, and 0 (curve 1), 20 (curve 2), 40 (curve 3), or 75 (curve 4) μM 2ME. The reaction mixtures were incubated 15 min at 37°C and chilled on ice, and then 10 μl of 2.5 mM GTP was added. Samples were transferred to 0°C cuvettes and baselines were established. At time zero, the temperature was set at 37°C and subsequently set at 10°C and 0°C at the times indicated by the vertical dashed lines. (*B*) Reaction mixtures contained 0.8 M monosodium glutamate (pH 6.6), 4% dimethyl sulfoxide, tubulin at 1.2 mg/ml, and 0 (curve 1), 1.0 (curve 2), 2.0 (curve 3), 3.0 (curve 4), or 4.0 (curve 5) μM 2ME. The reaction mixtures were incubated 15 min at 26°C and chilled on ice, and then 10 μl of 10 mM GTP was added. Samples were transferred to 0°C cuvettes and baselines were established. At time zero the temperature was set at 26°C and subsequently set at 0°C as indicated.

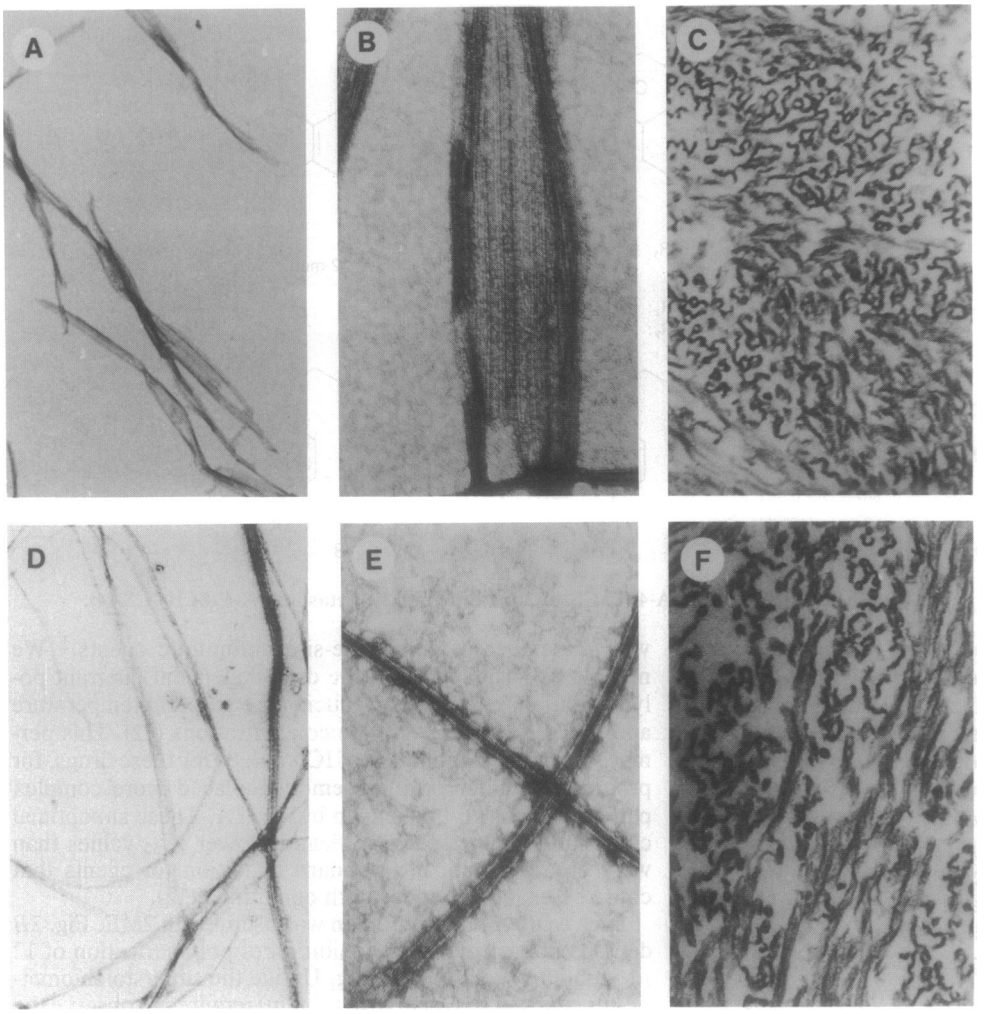

FIG. 3. Comparison of morphology of glutamate-induced polymer without (*A–C*) and with (*D–F*) 2ME. After 30 min at 37°C, aliquots from reaction mixtures containing the components described for Fig. 2*A*, including 40 μM (*F*) or 75 μM (*D* and *E*) 2ME, were placed on 200-mesh carbon-coated copper grids and stained with 0.5% uranyl acetate (*A*, *B*, *D*, and *E*), or the entire reaction mixture was centrifuged at 40,000 rpm for 20 min in a Beckman Ti50 rotor (*C* and *F*). Pellets were fixed in 10% (vol/vol) formalin and processed for thin-section electron microscopy (15). (*A*, ×28,100; *B* and *E*, ×112,500; *C* and *F*, ×67,500; *D*, ×22,500.)

CSA4 (compound 7), and H_2-CSA4 (compound 8). The plant-derived drugs yielded IC_{50} values ranging from 0.46 to 0.80 μM. The most potent estrogen derivative was 2ME (IC_{50}, 1.9 μM), followed by DES (IC_{50}, 2.4 μM). Estradiol had weak activity (IC_{50}, 30 μM; see ref. 6). A Br substituent at position C(2) enhanced the inhibitory effect of estradiol (IC_{50}, 4.5 μM), whereas a F substituent was without effect (IC_{50}, 27 μM). Analogs with a single methoxy group at position C(3) or C(4) were inactive, as were compounds with two methoxy groups at positions C(2) and C(3) or at C(3) and C(4). Moderate inhibitory activity was observed with 17-ethynylestradiol, but its 2-methoxy derivative was inactive, as were estriol and its 2-methoxy derivative. The 2-methoxy derivative of estrone had moderate inhibitory activity (IC_{50}, 9.0 μM), whereas the parent compound was inactive.

The control polymer (no 2ME) formed in the suboptimal condition was examined with the electron microscope. It closely resembled polymer formed in the standard system.

Structurally 2ME is similar to colchicine and related compounds, and DES inhibits colchicine binding to tubulin (2, 16). A concentration study (Fig. 4*A*) in which 2ME was compared to CSA4 (17) and H_2-CSA4 showed that 2ME was a weak inhibitor of the binding of [^{3}H]colchicine to tubulin. Greater than 50% inhibition occurred with 5 μM CSA4 or H_2-CSA4 as compared to 50% inhibition with about 40 μM 2ME. However, 2ME was significantly more effective than DES as an inhibitor of colchicine binding to tubulin, and it was more potent than any other estrogen derivative examined (Table 1; estrogens at 100 μM).

Detailed studies were performed to determine whether 2ME was a competitive inhibitor of colchicine binding, thus interacting at the colchicine site. In the Hanes format (18), these data yielded parallel curves at different 2ME concentrations (Fig. 4*B*), consistent with competitive inhibition (with noncompetitive inhibition the curves intercept on the abscissa). Replotted in the Dixon format (18), the data yield an apparent K_i value of 22 ± 2 μM (average ± SD) for 2ME

Table 1. Comparative effects of estrogenic steroid derivatives on tubulin polymerization and colchicine binding

Compound	Polymerization IC_{50}, μM	% of colchicine binding inhibition
2ME	1.9 ± 0.2	82 ± 2
DES	2.4 ± 0.4	30 ± 4
2-Bromoestradiol	4.5 ± 0.6	
2-Methoxyestrone	9.0 ± 1	57 ± 6
17-Ethynylestradiol	10 ± 2	50 ± 7
2-Fluoroestradiol	27 ± 6	
Estradiol	30 ± 6	38 ± 4
Podophyllotoxin	0.46 ± 0.02	
CSA4	0.53 ± 0.05	
H_2-CSA4	0.63 ± 0.03	
Colchicine	0.80 ± 0.07	

For polymerization, IC_{50} values were obtained three times for each drug, as described for Fig. 2*B*. Inactive compounds (IC_{50} > 40 μM) were estrone, 2-methoxy-17-ethynylestradiol, estriol, 2-methoxyestriol, estradiol 3-*O*-methyl ether, 2-methoxyestradiol 3-*O*-methyl ether, 4-methoxyestradiol, and 4-methoxyestradiol 3-*O*-methyl ether. For colchicine binding, reaction conditions were as in the text, and incubation was for 10 min at 37°C. Reaction mixtures contained 1.0 μM tubulin, 5% dimethyl sulfoxide, 2 μM [^{3}H]colchicine, and 100 μM inhibitor. Data are the average ± SD obtained in three experiments, except for 2-methoxyestrone (examined twice). Control value, 0.13 mol/mol of tubulin.

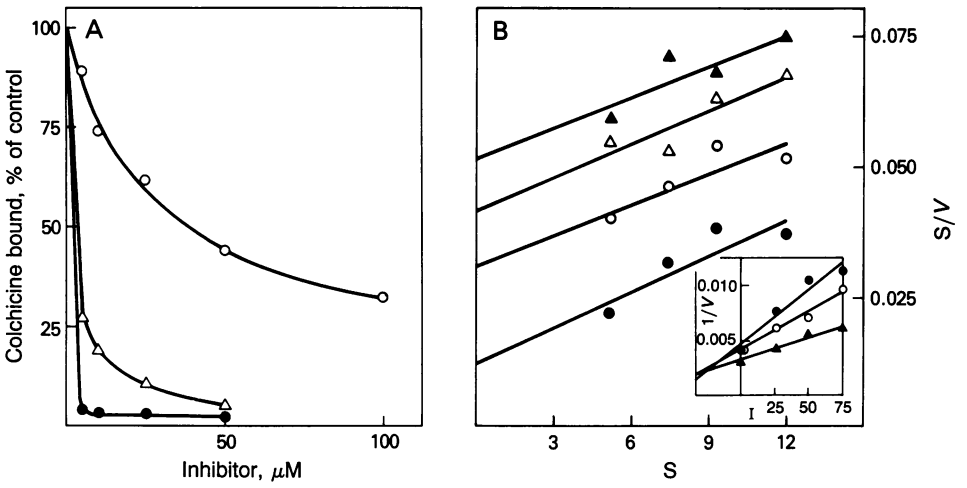

FIG. 4. Inhibition of colchicine binding to tubulin. (*A*) Comparison of 2ME to CSA4 and H_2-CSA4. Reaction mixtures contained 1.0 μM tubulin, 5% dimethyl sulfoxide, 5 μM [^{3}H]colchicine, and inhibitor as indicated. Incubation was 10 min at 37°C, reaching 30% of maximum. Control value, 0.17 mol/mol of tubulin. ○, 2ME; ●, CSA4; △, H_2-CSA4. (*B*) Hanes analysis of inhibition of colchicine binding by 2ME. Reaction conditions were as for *A*, with the indicated colchicine concentrations (S, in μM) and 2ME as follows: ○, none; ●, 25 μM; △, 50 μM; ▲, 75 μM. Units for *V* are pmol of colchicine bound per min per mg of tubulin. (*Inset*) Dixon analysis. Concentrations (μM) of 2ME (I) are on the abscissa. Colchicine was as follows: ●, 5.2 μM; ○, 7.3 μM; ▲, 12 μM. Units on ordinate are min·mg of tubulin per pmol of colchicine bound.

(Fig. 4*B Inset*; K_i value, average of three determinations). In previous experiments under the same reaction conditions, we obtained data with podophyllotoxin and CSA4 consistent with their being competitive inhibitors of colchicine binding, with apparent K_i values of 0.5 and 0.12 μM, respectively (17).

DISCUSSION

The results presented here demonstrate that the endogenous metabolite of estradiol, 2ME, inhibits tubulin polymerization. Seegers *et al.* (5) proposed that the antimitotic effects of estradiol were due to its metabolism to 2ME, but we are unaware of any demonstrated direct interaction of 2ME with tubulin.

It has long been postulated that the colchicine site of tubulin is a regulatory domain that interacts with endogenous substances affecting microtubule assembly. Efforts to define such compounds have been limited thus far to ill-defined proteins (19, 20). Our finding that 2ME binds to the colchicine site of tubulin and inhibits assembly provides evidence that endogenous molecules similar in size to colchicine could serve such functions. Our observations with 2ME suggest that other, perhaps unknown, steroid compounds could play important roles in microtubule assembly, possibly functioning as negative regulators.

Human serum levels of 2-methoxyestrogens range from 30 pM (adult males) up to 30 nM (pregnant females) (21), much lower than the concentrations required to inhibit tubulin polymerization. However, levels of 2ME inside cells are unknown, and specific cell types have not been described in which it accumulates. Insight into a more specific role for 2ME in the control of microtubules in interphase or mitotic cells requires such information.

Our results with 2ME in the standard system indicate that such regulatory roles may be subtle and involve incorporation of the small molecule into polymer. We have not demonstrated that 2ME enters the polymer formed in its presence, but both stability properties and morphology of polymer were altered with the drug. This probably results from copolymerization of tubulin–steroid complexes with unliganded tubulin, as can occur with colchicine (22). Small molecules incorporated into the microtubule network of cells could alter their stability, growth rates, or interactions with other macromolecules or small molecules.

With 2ME and other colchicine-site agents, relative activities as inhibitors of polymerization and of colchicine binding are not quantitatively concordant. Of the drugs examined here, there is little difference in the polymerization IC_{50} values of podophyllotoxin, CSA4, and H_2-CSA4, and 2ME is only 3- to 4-fold less active. The range of inhibition of colchicine binding is wider. When present at equimolar concentrations with [^{3}H]colchicine, inhibition with CSA4 was 95%, with podophyllotoxin was 85–90%, with H_2-CSA4 was 70–75%, and with 2ME was 10–15% (apparent K_i values of 22, 0.5, and 0.12 μM have been obtained for 2ME, podophyllotoxin, and CSA4, respectively). Experimental differences probably account for such discrepancies. With polymerization, a drug–protein preincubation was used, and complete inhibition occurred when the drugs were present in a substoichiometric concentration relative to tubulin. Even if all the drug were complexed to tubulin, the unliganded tubulin would still exceed the critical concentration for assembly in the suboptimal condition (tubulin at 0.33 mg/ml,[§] unpublished data). Thus, binding of drug to one tubulin molecule affects other tubulin molecules in the polymerization assay. The colchicine binding reaction is a stoichiometric assay, no preincubation was used, and the results reflect the relative affinities of colchicine and inhibitors for tubulin and their relative association and dissociation rates.

Among the steroid derivatives we examined for effects on tubulin assembly, none was as potent as 2ME. Important findings include a weak effect of estradiol, loss of activity if an additional methoxy substituent is attached to the aromatic A ring of estradiol, significant inhibitory activity of 2-methoxyestrone and 17-ethynylestradiol and inactivity of the 2-methoxy derivative of 17-ethynylestradiol.

The interaction of 2ME with the colchicine site may also provide structure–activity insights. This could assist in the rational design of additional chemotherapeutic agents. Excluding alkyl derivatives of benzimidazole methyl carbamate (23), previously described colchicine-site agents acting on mammalian tubulin, including DES, have two aromatic domains. Binding of aromatic drug domains to identical subsites of tubulin has not been demonstrated (for a speculative discussion, see ref. 12), but it may be useful to discuss these

[§]The critical concentration in the standard system is 0.10 mg/ml.

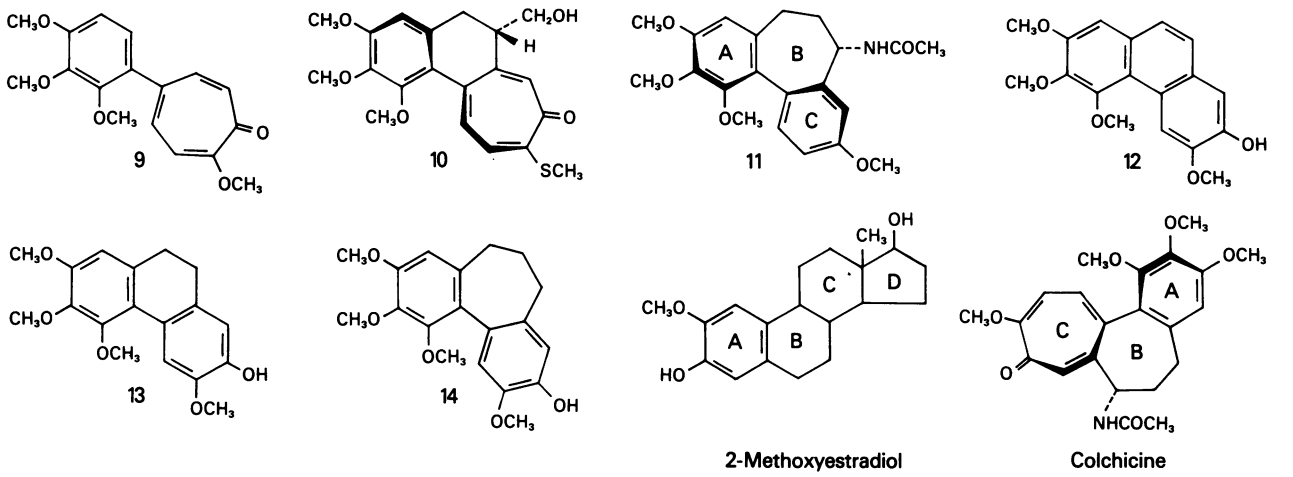

FIG. 5. Selected colchicine site compounds, with a comparison suggesting that the 2ME A ring corresponds to the colchicine C ring.

drugs in terms of colchicine as the model compound with ring A and ring C subsites on tubulin.

The interactions of highly active colchicine analogs with tubulin have been studied in some detail. The B ring and its C(7) substituent can be eliminated, for 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone (Fig. 5, compound **9**) is a potent inhibitor (24). The B ring can also be reduced to six members, with compound **10** as an example (25), or the C ring can be reduced to a six-member phenyl ring in the allocolchicinoid series, represented by compound **11** (26). In contrast, when the potent CSA4 is converted to a phenanthrene (compound **12**), antitubulin activity is lost (27). Loss of activity also occurs with the transition from H_2-CSA4 to the analogous dihydrophenanthrene (compound **13**) (27, 28). Expansion of the middle ring of the latter to seven members (compound **14**), forming an allocolchicinoid, restores activity (28). Thus, if a B ring is present with aromatic A and C rings, either the B ring or the C ring, but not both, can be six membered.

The activity of 2ME adds an unexpected element to these structure–activity observations: the steroid has structural analogy in its three conjoined six-member rings to the inactive phenanthrene and dihydrophenanthrene combretastatin analogs, but 2ME has only one aromatic ring. Perhaps loss of planarity and/or somewhat greater molecular size in the saturated six-member ring results in an altered configuration able to interact more effectively at the colchicine site, in comparison with the planar phenanthrene and slightly puckered dihydrophenanthrene structures.

Our finding that additional methoxy groups at positions 3 and 4 in the estradiol molecule lead to loss of antitubulin activity in the steroid derivatives suggests that the steroid A ring is homologous to the colchicine C ring (Fig. 5) and the CSA4 B ring rather than to the trimethoxybenzene rings. The latter may be homologous to the C ring/D ring complex of the steroid. This would be consistent with spectroscopic evidence suggesting the C ring of colchicine interacts by base stacking with a tryptophan residue of tubulin (29). If this conclusion is correct, then a search for more potent antitubulin steroid derivatives should focus on modifications in the C ring/D ring region.

In summary, the antimitotic activity of 2ME probably results from its binding at the colchicine site of tubulin. The estrogen metabolite inhibits polymerization at substoichiometric concentrations under a suboptimal reaction condition. Although the effects of the steroid derivative are weaker than those of many antimitotic agents, 2ME is the most potent endogenous inhibitor of tubulin polymerization produced by mammalian tissues yet described. This compound or related steroid derivatives may play a significant role in modulating microtubule assembly and function in mammals.

This paper is dedicated to Dr. Arnold Brossi on the occasion of his 70th birthday. R.J.D. is a Howard Hughes Medical Institute physician research fellow.

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