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A Steroid Derivative with Paclitaxel-Like Effects on Tubulin Polymerization

PASCAL VERDIER-PINARD, ZHIQIANG WANG, ARASAMBATTU K. MOHANAKRISHNAN, MARK CUSHMAN, and ERNEST HAMEL

Laboratory of Drug Discovery Research and Development, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland (P.V.-P., E.H.); and Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana (Z.W., A.K.M., M.C.)

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ABSTRACT

The endogenous estrogen metabolite 2-methoxyestradiol has modest antimitotic activity that may result from a weak interaction at the colchicine binding site of tubulin, but it nevertheless has in vivo antitumor activity. Synthetic efforts to improve activity led to compounds that increased inhibitory effects on cell growth, tubulin polymerization, and binding of colchicine to tubulin. This earlier work was directed at modifications in the steroid A ring, which is probably analogous to the colchicine tropolonic C ring. One of the most active analogs prepared was 2-ethoxyestradiol (2EE). We report here that different modifications in the steroid B ring of 2EE yield compounds with two apparently distinct modes of action. Simple expansion of the B ring to seven members resulted in a compound comparable to 2EE in its ability to inhibit tubulin polymerization and colchicine binding to tubulin. Acetylation of the hydroxyl groups in this analog and in 2EE essentially abolished these inhibitory properties. The introduction of a ketone functionality at C6, together with acetylation of the hydroxyls at positions 3 and 17, produced a compound with activity similar to that of paclitaxel, in that the agent enhanced tubulin polymerization into polymers that were partially stable at 0°C. The acetyl group at C17, but not that at C3, was essential for this paclitaxel-like activity.

Tubulin, the building block of microtubules, is the target of many antimitotic drugs (for a review, see Hamel, 1996). Three major pharmacological sites are present on tubulin: the colchicine site, the vinca alkaloid domain, and the taxoid site. The latter is fully expressed only in tubulin polymers with a substructure of well defined protofilaments (Parness and Horwitz, 1981; Takoudju et al., 1988). Among antimitotic agents that appear to bind at the colchicine site are synthetic analogs of estradiol, such as diethylstilbestrol and the major endogenous metabolite of estradiol, 2-methoxyestradiol (2ME; structure in Fig. 1, with comparison with the structure of colchicine; D'Amato et al., 1994). 2ME also has significant antiangiogenic properties and in vivo antitumor activity (Fotsis et al., 1994; Klauber et al., 1997). Based on the ability of 2ME to inhibit the binding of colchicine to tubulin, we proposed that its A ring might bear structural analogy to the C ring of colchicine.

These findings have led to synthetic efforts to prepare steroid derivatives with better activity than 2ME. Among the compounds we prepared, 2-ethoxyestradiol (2EE) had greater inhibitory effects on tubulin polymerization and was 10-fold more cytotoxic than 2ME (Cushman et al., 1995). Taking another tack, Miller et al. (1997) prepared seven-member tropolonic A ring analogs of 2ME to enhance the analogy to the C ring of colchicine and found several of these to be highly active inhibitors of tubulin assembly. Because allocolchicinoids, with a seven-member B ring but six-member C ring, such as compound 1 (Iorio, 1984), are often more active than the corresponding colchicinoids (Kang et al., 1990), we decided to synthesize steroid derivatives with an expanded B ring, with the two isomers of compound 2 being our ultimate target. We were encouraged to find the intermediate compound 5 (see reaction scheme in Fig. 2) active as an inhibitor of assembly, but ketone 8, representing the next step in the synthesis, after deprotection of 7, was inactive.

In our previous work, we often analyzed protected estradiol analog intermediates bearing acetyl groups at positions C2 and/or C17. Invariably, these compounds had minimal or no effect on tubulin polymerization (Cushman et al., 1995, 1997). When a similar screening assay was performed with compound 7 (Fig. 2), the diacetate of 8, we observed a stimulation of assembly qualitatively similar to that which occurs with the potent anticancer drug paclitaxel (Schiff et al., 1979; Hamel et al., 1981; Schiff and Horwitz, 1981). This study describes our initial analysis of this observation, as well as the expected inhibitory effects of compound 5. Finally, we

ABBREVIATIONS: 2ME, 2-methoxyestradiol; 2EE, 2-ethoxyestradiol; MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethanesulfonate.

prepared and evaluated the two monoacetate analogs 9 and 10 (Fig. 2) and demonstrated that 9 was inactive with tubulin but that 10 was almost as active as 7.

Experimental Procedures

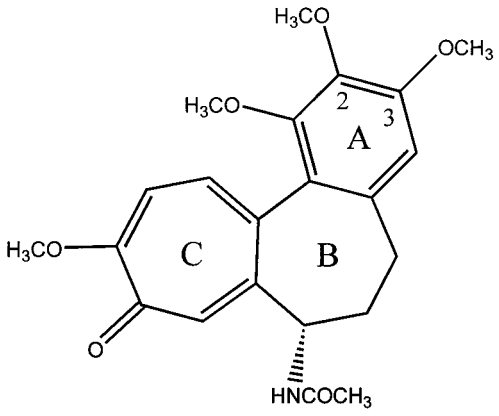
Materials. Electrophoretically homogeneous bovine brain tubulin and heat-treated microtubule-associated proteins (MAPs; Hamel and Lin, 1984), 2EE (Cushman et al., 1995), and compound 3 (Cushman et al., 1997) were prepared as described previously. The synthesis of compounds 5 to 10 was performed as outlined in Fig. 2, with details of their preparation to be presented elsewhere. GTP, repurified by anion exchange chromatography, was obtained from Sigma Chemical Co. (St. Louis, MO). 2ME was obtained from Aldrich Chemical (Milwaukee, WI). [^{3}H]Colchicine was purchased from DuPont-New England Nuclear (Boston, MA). Paclitaxel and [^{3}H]paclitaxel were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. Estramustine phosphate was a generous gift from Kabi Pharmaceuticals (Helsingborg, Sweden).

Methods. Tubulin polymerization was followed turbidimetrically at 350 nm in Gilford model 250 spectrophotometers equipped with electronic temperature controllers. Temperature in the cuvettes rises at ~0.5°C/s and falls at ~0.1°C/s in the 0–37°C range. Specific reaction conditions are described for the individual experiments.

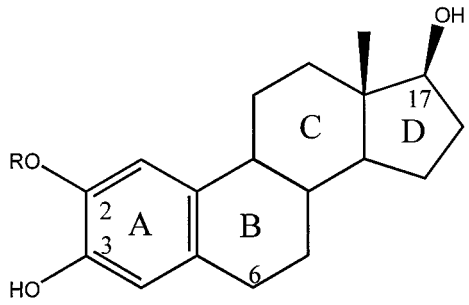
Results

Inhibition of Tubulin Polymerization by Compound 5. The newly synthesized steroid derivatives described here were all examined for inhibitory effects on tubulin polymerization and on the binding of [^{3}H]colchicine to tubulin. Only compound 5 had significant activity, and in Table 1 we summarize our findings, together with simultaneously obtained data with 2ME and 2EE. [The compound resulting from deacetylation of compound 3, as previously reported (Cushman et al., 1997), does inhibit tubulin assembly and colchicine binding, with activities differing little from those of 2ME.] Estramustine phosphate was also examined and was found to be much less potent as an inhibitor of tubulin polymerization, in agreement with the results of Dählof et al. (1993). Estramustine phosphate also interacts with MAPs [see Tew et al. (1992) for a review]. Expanding the steroid B ring to seven members failed to improve on the activity observed with 2EE in either assay. As an inhibitor of assembly, compound 5 was closer in activity to 2EE, but as an inhibitor of colchicine binding, its activity was closer to that of 2ME. We emphasize that compound 6, the diacetate of 5, neither inhibited colchicine binding to tubulin nor significantly affected tubulin assembly. Estramustine phosphate had no effect on the binding of [^{3}H]colchicine to tubulin, which is consistent with reports of others (Laing et al., 1997; Panda et al., 1997) that this agent may have a distinct binding site on tubulin.

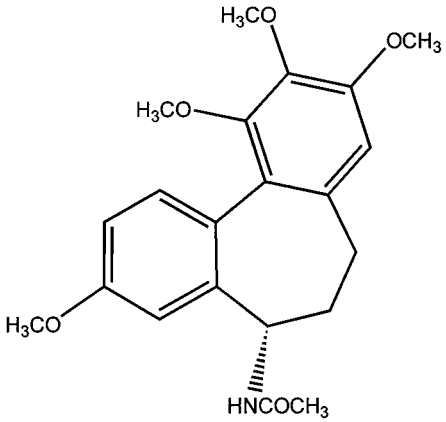
Stimulation of Tubulin Assembly Induced by Glutamate by Compounds 7 and 10. In Fig. 3A, the effect of compound 7 on glutamate-induced assembly is presented.



Colchicine



2ME: R = CH_3-
2EE: R = CH_3CH_2-



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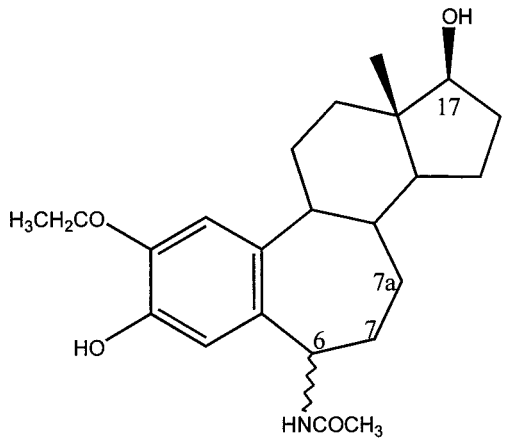

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Fig. 1. Structures of colchicine, 2ME, 2EE, and related compounds. The chemical names of compounds 1 and 2 are as follows: compound 1, *N*-acetylcolchinol *O*-methyl ether; compound 2, 6-acetamido-2-ethoxy-B-homo-estra-1,3,5(10)-trien-3,17β-diol.

Increasing amounts of the agent, up to ~10 μM, which is near stoichiometric with the 12 μM tubulin concentration, resulted in progressively more rapid reactions that all reached the same turbidity plateau. The polymer formed was highly stable at 0°C at higher concentrations of compound 7. Deacetylation of compound 7 to yield compound 8 eliminated this stimulatory activity, as shown by curve 6 in Fig. 3A. The reaction mixture represented by curve 6 contained 40 μM compound 8.

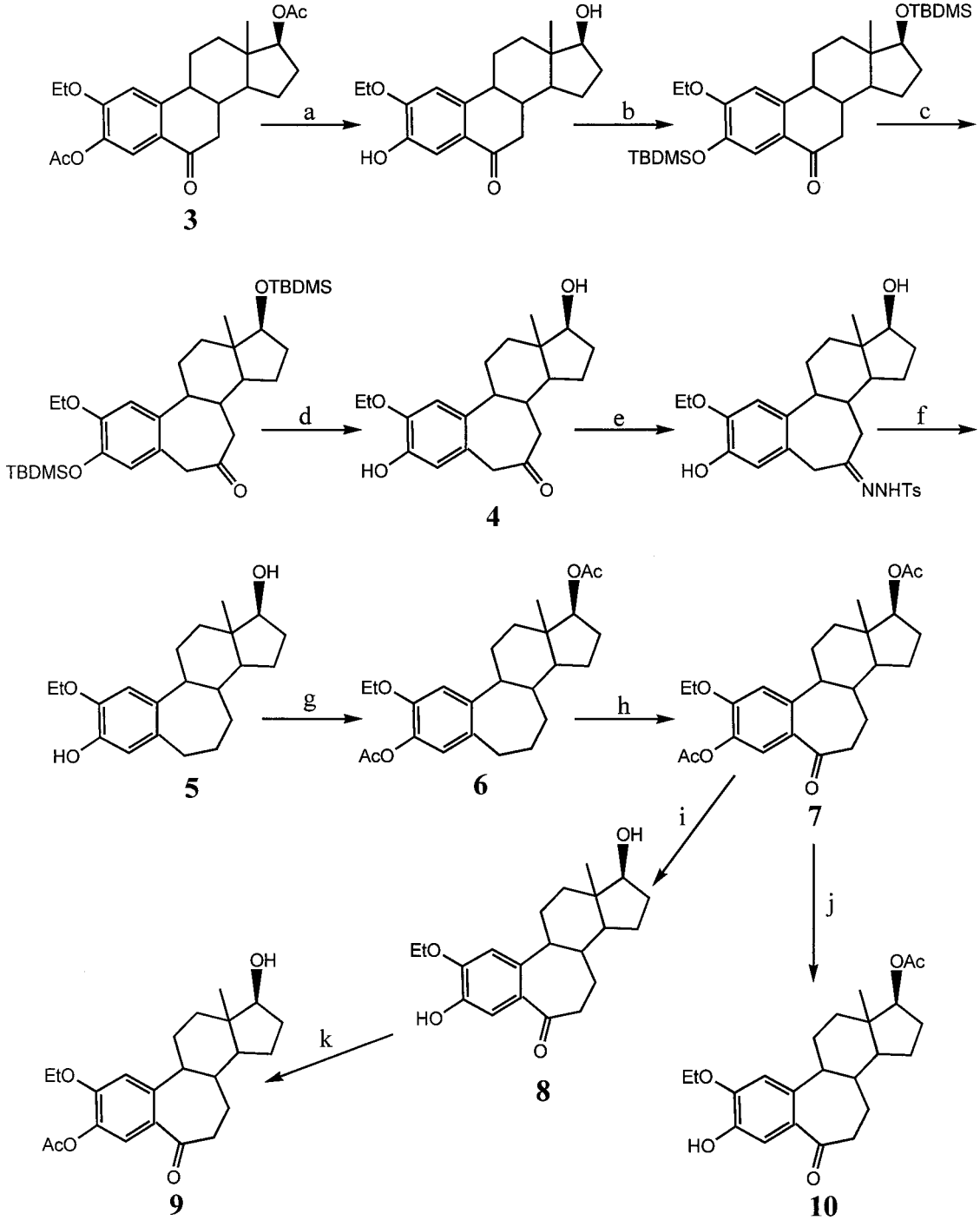

Fig. 2. Synthetic pathway to diacetate 7 and monoacetates 9 and 10. The reagents and reaction conditions indicated by the letters above the arrows are as follows: a, KOH, methanol, 4 h at room temperature; b, *tert*-butyldimethylsilyl chloride, imidazole, *N*,*N*′-dimethylformamide, 18 h at room temperature; c, first CH_2Br_2, lithium diisopropylamide, tetrahydrofuran, −78°C, 3 h, and then butyllithium added and the temperature increased to 0°C, 2.5 h; d, tetrabutylammonium fluoride, tetrahydrofuran, 4 h at room temperature; e, tosylhydrazine, methanol, 24 h at room temperature; f, first catecholborane, $CHCl_3$, and then sodium acetate, water, refluxed for 3 h; g, acetic anhydride, pyridine, 24 h at room temperature; h, CrO_3, acetic acid, 12–14°C for 40 min; i, KOH, methanol, 3.5 h at −5°C to room temperature; j, $KHCO_3$, methanol, 65°C for 1 h; and k, 1-acetyl-1*H*-1,2,3-triazolo[4,5-*b*]pyridine, aqueous NaOH, tetrahydrofuran, 1.5 h at room temperature. Ac, acetyl; Et, ethyl; TBDMS, *tert*-butyldimethylsilyl; Ts, tosyl. The chemical names of compounds 7 to 10 are as follows: compound 7, 3,17β-diacetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-triene; compound 8, 2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-trien-3,17β-diol; compound 9, 3-acetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-trien-17β-ol; and compound 10, 17β-acetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5(10)-trien-3-ol.

Compounds 9 and 10 were synthesized to determine whether both acetyl groups were required for this ability to enhance polymer formation. The addition of a single acetyl at position C3 yielded the inert compound 9, but the addition of a single acetyl group at C17 yielded compound 10 with activity only slightly less than that of compound 7 (Fig. 3B). Much less dramatic paclitaxel-like effects were also observed with compound 3 and the ketone diacetate of compound 4, with these compounds at 40 μM having effects less than those observed with 10 μM compound 7 or 10. Compounds 7 and 10 had no inhibitory effect on the binding of [^{3}H]colchicine to tubulin.

TABLE 1

Inhibition of tubulin polymerization and the binding of [^{3}H]colchicine to tubulin by compound 5, 2ME, and 2EE

Tubulin polymerization and colchicine-binding assays were performed as described previously (D'Amato et al., 1994). In the polymerization assay, 12 μM tubulin was preincubated with varying drug concentrations for 15 min at 26°C before the addition of GTP. The assembly reactions were followed turbidimetrically for 20 min at 26°C, and drug concentrations required for 50% reduction in the plateau turbidity reading were determined. In the colchicine-binding assays, reaction mixtures contained 1.0 μM tubulin, 5.0 μM [^{3}H]colchicine, and inhibitors, if present, at 50 μM. Incubation was for 10 min at 37°C. Previous studies have shown that at this time point, the colchicine-binding reaction was about 50% complete in the control reaction mixtures under the reaction conditions used. All experiments were performed twice, and standard deviations are shown. In the colchicine-binding experiments, the individual assays were also performed with duplicate samples.

Compound	Inhibition of Polymerization IC_{50}	Inhibition of Colchicine Binding
	μM ± S.D.	*% inhibition ± S.D.*
5	1.3[a]	56 ± 7
2ME	2.2 ± 0.3	60 ± 10
2EE	1.0 ± 0.07	87 ± 4
Estramustine phosphate	120 ± 20	0[a]

[a] Same value obtained in both experiments.

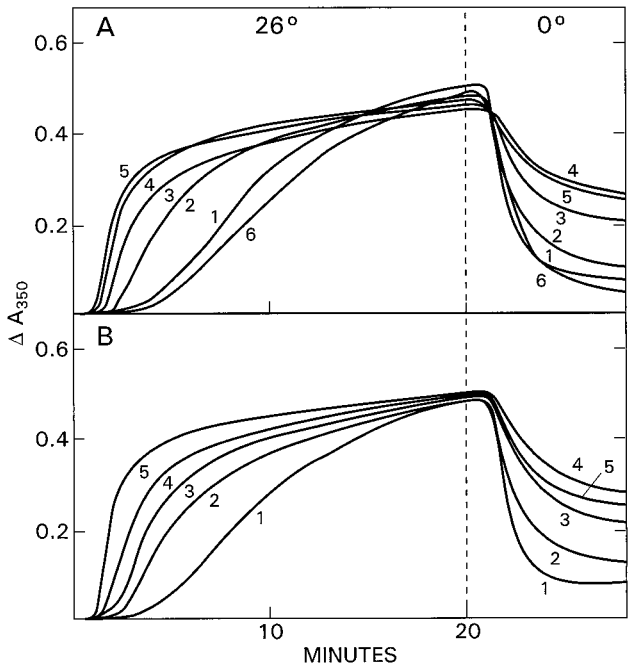

Fig. 3. Enhancement of glutamate-induced tubulin assembly by compounds 7 (A) and 10 (B). Each reaction mixture contained 0.8 M monosodium glutamate (pH 6.6 with HCl in 2 M stock solution), 0.4 mM GTP, 12 μM (1.2 mg/ml) tubulin, 4% (v/v) DMSO, and drug as indicated. Baselines were established at 0°C, and at time zero the temperature controller was set at 26°C. At the time indicated by the dashed line, the temperature controller was set at 0°C. A, curve 1, no drug; curve 2, 2 μM compound 7; curve 3, 5 μM compound 7; curve 4, 10 μM compound 7; curve 5, 40 μM compound 7; and curve 6, 40 μM compound 8. B, curve 1, no drug; curve 2, 2 μM compound 10; curve 3, 5 μM compound 10; curve 4, 10 μM compound 10; and curve 5, 40 μM compound 10.

Stimulation by Compounds 7 and 10 of MAP-Induced Tubulin Assembly. As shown in Fig. 4, in experiments with the optimum amount of our current MAP preparation (0.75 mg/ml MAP with 1.0 mg/ml tubulin), compounds 7 and 10 enhanced tubulin assembly at 25°C, although their activities appeared to be quantitatively lower than had been the case in the glutamate reaction condition. A progressive increase in activity with up to 40 μM agent was seen with both compounds (data presented in full only for compound 7), and at all concentrations compound 10 was less stimulatory than 7 (Fig. 4 presents data with compound 10 only at 10 μM). In addition, Fig. 4 demonstrates the effect of 10 μM paclitaxel on the assembly reaction. With a temperature jump directly from 0–25°C, the assembly reaction with paclitaxel was much more extensive than that with compound 7 (not shown). In part, this could be attributed to assembly stimulated by paclitaxel that occurred before temperature equilibration (Grover et al., 1995), and this could be clearly demonstrated by adding a 10°C step to the reaction sequence, as was done in the experiment presented in Fig. 4.

It was possible that compounds 7 and 10 were not actually acting like paclitaxel in enhancing microtubule assembly and in stabilizing the microtubules formed in the course of the turbidity studies shown in Fig. 4 but rather that these agents were causing the formation of polymers of aberrant morphology and temperature stability different from microtubules, such as occurs with vinca alkaloids (Himes, 1991) or curacin A (Hamel et al., 1995). We therefore examined polymer morphology in the electron microscope. With compound 7, abundant microtubules were formed that were indistinguishable from those formed in the absence of drug. Even more important, when the reaction mixtures were returned to 0°C for 15 min, no microtubules were visualized on grids prepared from the reaction mixture without drug. In the presence of 40 μM

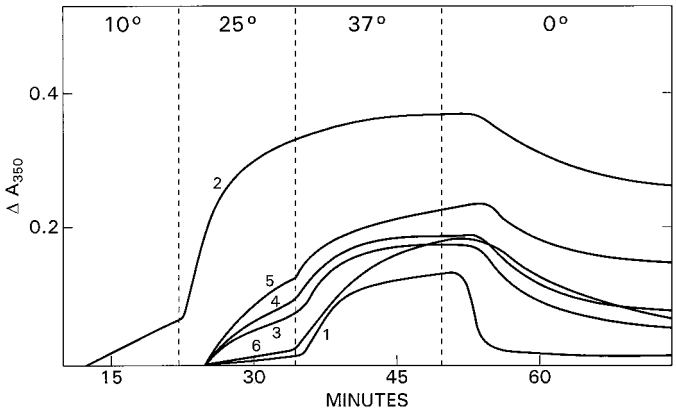

Fig. 4. Enhancement of MAP-induced tubulin assembly by compounds 7 and 10. Each reaction mixture contained 0.1 M 2-(*N*-morpholino)ethanesulfonate (MES) (pH 6.9 with NaOH in 0.5 M stock solution), 0.1 mM GTP, 10 μM (1.0 mg/ml) tubulin, 0.75 mg/ml heat-treated MAPs, 4% DMSO, and drug as indicated. Baselines were established at 0°C, with drug added last, and the cuvette contents were observed for 10 min. There was no change in turbidity in any reaction mixture (not shown). At this point, the temperature controller was set at 10°C, and subsequent temperature changes, as indicated, were made at the times indicated by the vertical dashed lines. Curve 1, no drug; curve 2, 10 μM paclitaxel; curve 3, 10 μM compound 7; curve 4, 20 μM compound 7; curve 5, 40 μM compound 7; and curve 6, 10 μM compound 10.

7 (Fig. 5) or 10 μM paclitaxel (not shown), abundant cold stable microtubules were observed.

When either MAPs or GTP was omitted from the reaction mixture, with 10 μM tubulin no reaction occurred with either no drug or 40 μM compound 7. In contrast, with 10 μM paclitaxel, a reaction was observed in both cases (cf. Hamel et al., 1981; Grover et al., 1995).

Quantification of Relative Effects of Paclitaxel and Compound 7 on Tubulin Polymerization. It is clear from the data of Fig. 4 that the activities of 7 and 10 are significantly less than that of paclitaxel. We wanted to put some quantitative measurement on the difference. We found, however, that we could demonstrate only minimal inhibition by compound 7 of the binding of [^{3}H]paclitaxel to tubulin polymer, nor was compound 7 active in room temperature glutamate-dependent assay systems (Hamel et al., 1999) where no polymerization reaction occurs in the absence of drug. We therefore returned to the MAP/GTP system, and we examined the effect of 10 μM compound 7 on 40 μM tubulin. We did observe assembly without MAPs but not without GTP. We therefore decided that measuring the critical concentration with and without MAPs with paclitaxel and compound 7 should provide some idea of the comparative activity of these agents. With MAPs, a constant weight ratio to tubulin of 1:3 was used, because the suboptimal concentration of MAPs caused greater differences in the critical concentrations obtained. The data are presented in Fig. 6, which yield critical concentrations, obtained at 30°C, of 1.4, 0.06, and 0.50 mg/ml for MAP- and GTP-dependent assembly without drug and in the presence of 10 μM paclitaxel or 10 μM 7, respectively, and 0.20 and 1.4 mg/ml for GTP-dependent, MAP-independent assembly with 10 μM paclitaxel or 10 μM 7 (no reaction occurred without drug at tubulin concentrations up to 4 mg/ml).

At first glance, these data suggest that the affinity of paclitaxel for tubulin polymers is only 7- to 8-fold greater than that of compound 7, but this method may significantly underestimate the quantitative difference between the two compounds. We previously observed with paclitaxel analogs that differences between compounds are magnified in restrictive reaction conditions and minimized in favorable reaction conditions (Grover et al., 1995; Kingston et al., 1998). Analogously, in recently reported studies, we found sarcodictyin A to be significantly less potent than paclitaxel in its interactions with tubulin under restrictive reaction conditions, and it only weakly inhibited the binding of radiolabeled paclitaxel to tubulin polymer (Hamel et al., 1999). However, under a favorable reaction condition (at room temperature), the quantitative difference between paclitaxel and sarcodictyin A was only 2.5-fold. Compound 7 was inactive in this room temperature assay. We therefore also determined the critical concentration with 10 μM sarcodictyin A under the reaction conditions shown in Fig. 6B, obtaining a value of 1.0 mg/ml in the GTP only system (data not presented). Relative order of

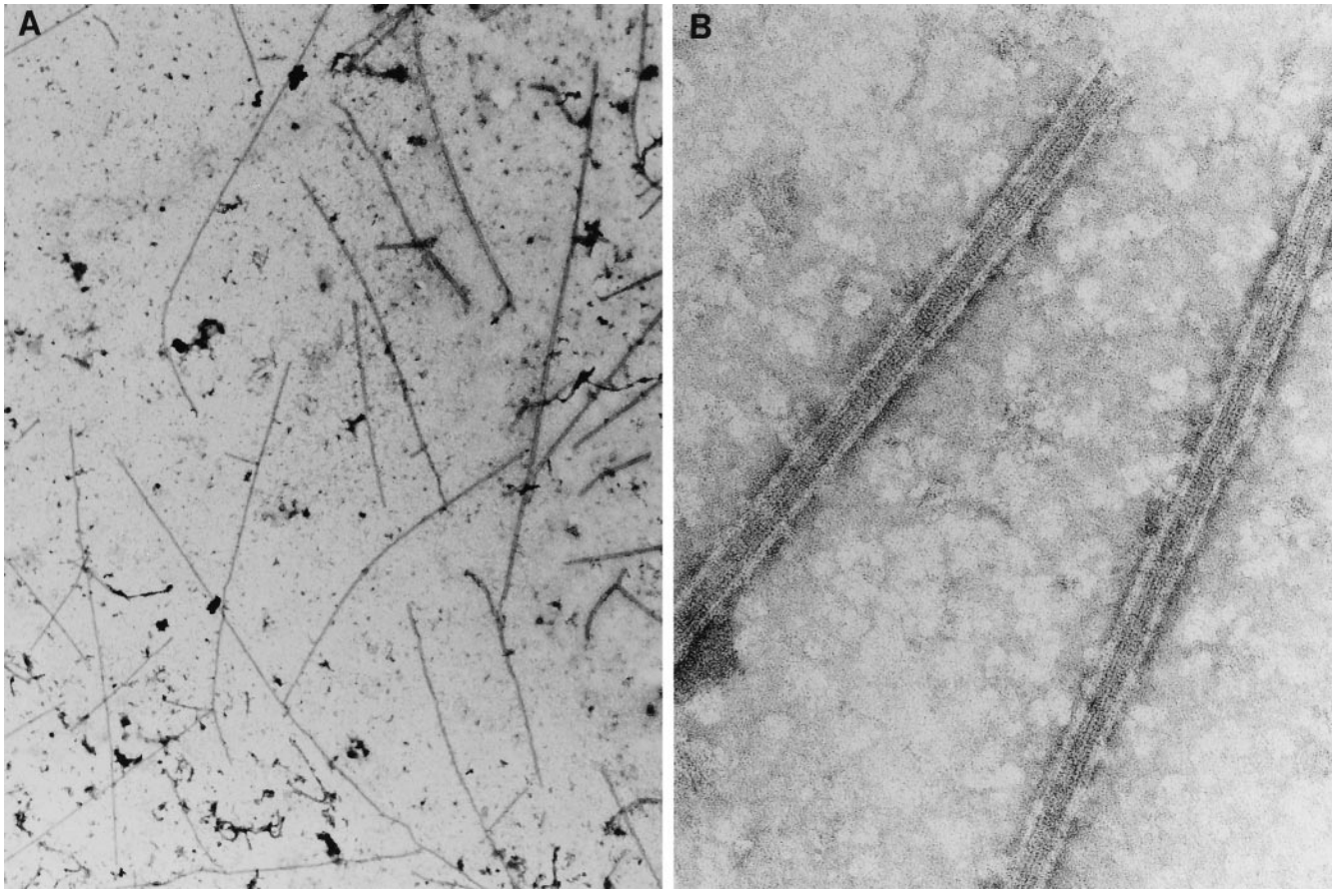

Fig. 5. Cold-stable microtubules formed in the presence of compound 7. The reaction mixture was identical with that indicated by curve 5 in Fig. 4. Polymer was assembled during a 20-min incubation at 37°C, and the reaction mixture was then chilled at 0°C for 20 min. An aliquot was placed on a carbon-coated, Formavar-treated, 200-mesh copper grid. The sample was washed off by a stream of droplets of 0.5% uranyl acetate, with excess stain wicked from the grid with filter paper. The grid was examined in a Zeiss model 10CA electron microscope (Magnification: A, 9,900×; B, 159,000×).

compound activity thus persists, with sarcodictyin A being ~5-fold less active than paclitaxel and 40% more active than 7, but this critical concentration assay probably overestimates the biochemical activity of less active taxoid-mimetic compounds.

Discussion

We originally evaluated the interaction of 2ME with tubulin because Seegers et al. (1989) found that the compound caused accumulation of mitotic MCF-7 cells with malformed spindles. We observed little effect of 2ME on MAP-dependent tubulin assembly, but with glutamate plus Mg^{2+}, assembly rate was reduced with formation of a morphologically unaltered polymer with enhanced temperature stability. The elimination of Mg^{2+} converted 2ME to a pure inhibitor of assembly (D'Amato et al., 1994). Studies with [^{3}H]2ME showed that binding of 2ME to unpolymerized tubulin was strongly inhibited by colchicine site drugs. Binding of 2ME to glutamate polymer occurred in a 1:1 stoichiometry with tubulin in a reaction minimally inhibited by colchicine-site drugs, suggesting a distinct binding site for 2ME on polymer or that 2ME binds in a site inaccessible to colchicine in polymer (Hamel et al., 1996). Binding of [^{3}H]2ME to polymer was minimally inhibited by paclitaxel (E.H., unpublished data).

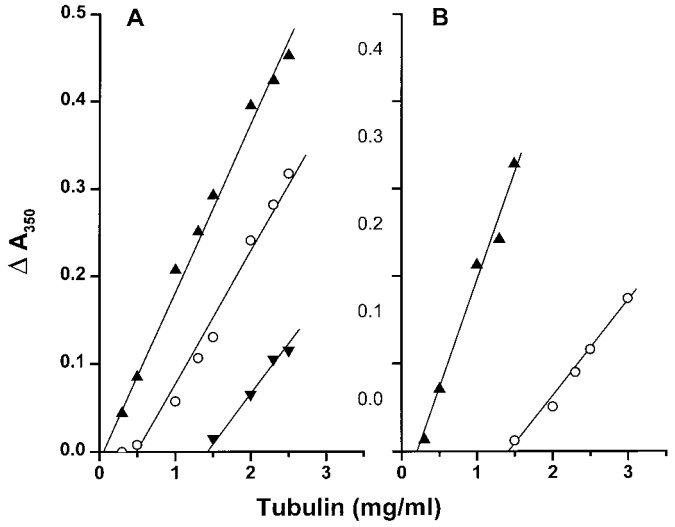

Fig. 6. Critical concentrations of tubulin with paclitaxel and compound 7 in reaction mixtures containing MAPs plus GTP (A) or GTP only (B). Reaction mixtures (0.25 ml) contained 0.1 M MES (pH 6.9), 100 μM GTP, 4% DMSO, the indicated tubulin concentration (with heat-treated MAPs in 1:3 weight ratio to the tubulin, A only), and either 10 μM paclitaxel (▲), 10 μM compound 7 (○), or no drug (▼). Cuvette contents were equilibrated at 0°C, drugs were added, temperature was jumped to 30°C (~1 min), and the turbidity changes after a 20-min incubation were measured.

In subsequent synthetic studies, enhanced cytotoxicity correlated well with enhanced inhibition of both glutamate-induced assembly and colchicine binding, with one of the more active analogs being 2EE (Cushman et al., 1995, 1997). In these studies, acetylation of the C3 and/or C17 hydroxyl groups led to a substantial loss of activity, and this finding continues with the contrast between the inhibition obtained with compound 5 and the inactivity of compound 6. Differing from these observations with steroid derivatives that inhibit tubulin polymerization are our findings with compounds 7 to 10. The diacetate compound 7 had taxoid-mimetic properties in both glutamate- and MAP-induced assembly reactions, whereas the dialcohol compound 8 was inactive. Removal of the C17 acetyl group yielded the inactive compound 9, but activity was largely retained after removal of the C3 acetyl group (compound 10). Figure 7 summarizes our current knowledge of structure-activity relationships of compounds related to 2ME.

The taxoid-mimetic properties of compounds 7 and 10 included more rapid induction of polymer at reduced temperatures, formation of microtubules stable to disassembly at 0°, reduction in the tubulin critical concentration, and polymer formation in 0.1 M MES without MAPs. In contrast to paclitaxel (Hamel et al., 1981; Schiff and Horwitz, 1981; Grover

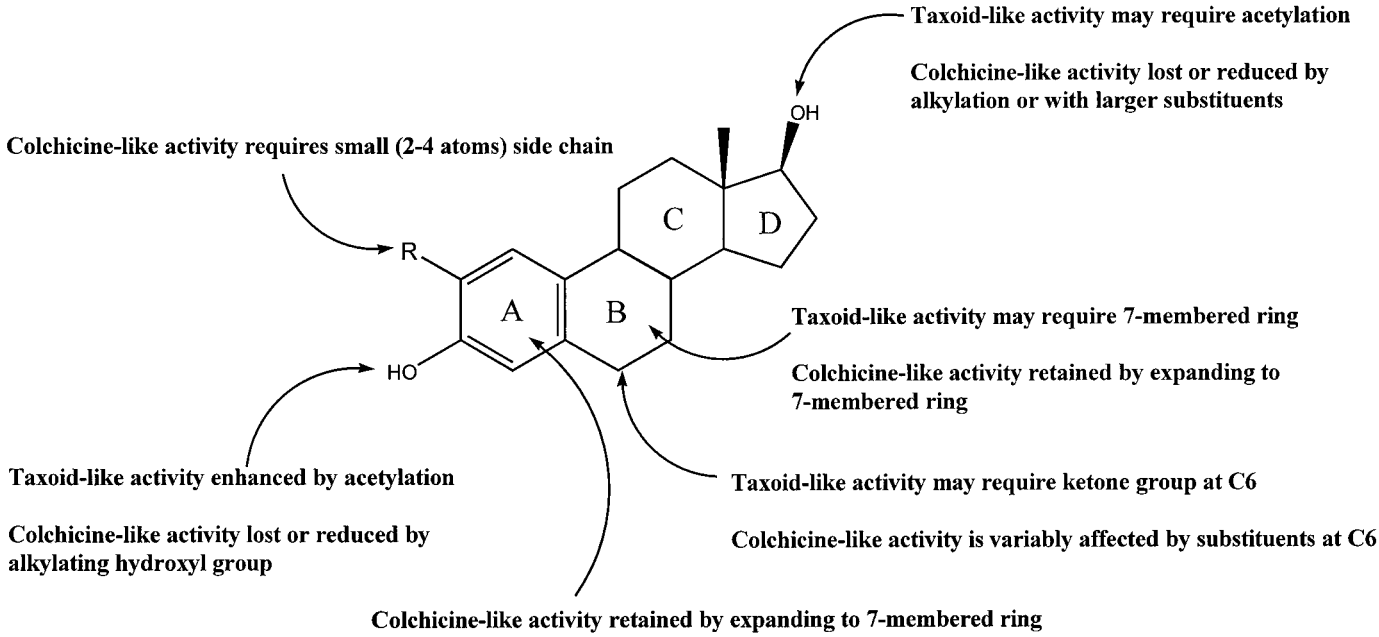

Fig. 7. Current summary of structure-activity relationships among steroid derivatives related to 2ME.

et al., 1995), we did not observe tubulin assembly in the absence of GTP with compound 7 or 10. Only 20% inhibition of [^{3}H]paclitaxel binding to tubulin polymer by compound 7 was observed, suggesting binding in the paclitaxel site on tubulin polymers.

Photoaffinity analogs of paclitaxel react covalently with β-tubulin peptides 1-31 (Rao et al., 1994) and 217-231 (Rao et al., 1995) and with β-Arg282 (Rao et al., 1999), observations consistent with the location of docetaxel in the model of tubulin derived by Nogales et al. (1998) from zinc-induced tubulin polymer. Direct photoactivation of colchicine by illumination of the tubulin-colchicine complex at 350 nm (absorbance maximum caused by the tropolone C ring) resulted in cross-links between radiolabeled colchicine and β-tubulin peptides 1-36 and 214-241 (Uppuluri et al., 1993). The analogy we proposed between the colchicine C ring and the 2ME A ring (D'Amato et al., 1994) is supported by the structure-activity work of Miller et al. (1997), who synthesized steroid derivatives with tropolonic A rings with enhanced antitubulin activity. These findings are thus highly suggestive that there may be at least some overlap between the site at which paclitaxel binds on tubulin polymer and the site the colchicine C ring occupies in unpolymerized tubulin.

In contrast to the colchicine direct photoaffinity study, a thiocolchicine derivative with a chemically reactive chloroacetyl group replacing the C3-methoxy reacted preferentially with β-Cys354 (Bai et al., 1996), whereas replacing the C2-methoxy group with the chloroacetyl group yielded an analog that reacted with both β-Cys354 and β-Cys239 (R. Bai and E. Hamel, in preparation). This seems to place the A ring of colchicine between these residues. Downing and Nogales (1998) were able to use these observations to place colchicine within their model in a region of tubulin distinct from that occupied by docetaxel, but it remains possible that the binding sites for the two drugs possess some common structural elements. Furthermore, there must be some significant conformational change in tubulin on its polymerization, because colchicine will not bind to microtubules (Wilson and Meza, 1973; Lee et al., 1974) and paclitaxel will not bind to unpolymerized tubulin (Parness and Horwitz, 1981; Takoudju et al., 1988).

We have not observed significant cytotoxicity of compound 7 or 10 in any cell line studied. This is probably not surprising, because the sarcodictyins also have feeble effects on cell growth (Ciomei et al., 1997; Hamel et al., 1999). However, it is likely that still more active steroid derivatives can be synthesized or discovered in existing compound collections, and we hope that our findings will encourage such efforts.

Finally, it has been long postulated that drug-binding sites on tubulin represent sites for endogenous molecules that regulate cellular microtubule structure and function. The realization that low concentrations of antimitotic drugs can have dramatic effects on microtubule dynamics (Wilson and Jordan, 1995) strengthens this concept. It is thus of interest that relatively minor structural changes in steroid derivatives can have opposite effects on tubulin assembly and polymer stability and, possibly, differing effects on microtubule dynamic properties. Perhaps known or still undiscovered steroids play important roles in regulating microtubule structure and function in eucaryotic cells.

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Send reprint requests to: Dr. E. Hamel, P.O. Box B, Building 469, Room 237, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702. E-mail: hamele@dc37a.nci.nih.gov