Estramustine Binds a MAP-1–like Protein to Inhibit Microtubule Assembly in Vitro and Disrupt Microtubule Organization in DU 145 Cells

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Abstract. The twofold purpose of the study was (*a*) to determine if a MAP-1–like protein was expressed in human prostatic DU 145 cells and (*b*) to demonstrate whether a novel antimicrotubule drug, estramustine, binds the MAP-1–like protein to disrupt microtubules. SDS-PAGE and Western blots showed that a 330-kD protein was associated with microtubules isolated in an assembly buffer containing 10 μM taxol and 10 mM adenylylimidodiphosphate. After purification to homogeneity on an A5m agarose column, the 330-kD protein was found to promote 6 S tubulin assembly. Turbidimetric (A_{350}), SDS-PAGE, and electron microscopic studies revealed that micromolar estramustine inhibited assembly promoted by the 330-kD protein. Similarly, estramustine inhibited binding of the 330-kD protein to 6-S microtubules independently stimulated to assemble with taxol. Immunofluorescent studies with β-tubulin antibody (27B) and MAP-1 antibody (MI-AI) revealed that 60 μM estramustine (*a*) caused disassembly of MAP-1 microtubules in DU 145 cells and (*b*) removed MAP-1 from the surfaces of microtubules stabilized with 0.1 μM taxol. Taken together the data suggested that estramustine binds to a 330-kD MAP-1–like protein to disrupt microtubules in tumor cells.

THE functional role of microtubule-associated proteins is poorly understood. Based on in vitro studies it has been proposed that MAPs might regulate microtubule assembly and disassembly in cells. Different classes of MAPs have been identified suggesting that different MAPs might provide for specialized functions associated with subclasses of microtubules (Vallee et al., 1986). In this regard immunofluorescent and immunoblotting studies of certain MAPs (tau, MAP-1, MAP-2, the 210–215 kD tumor cell MAPs) have shown organ, tissue, and cell specificity (Binder et al., 1986; Drubin et al., 1986; Matus and Riederer, 1986; Olmsted et al., 1986; Bloom et al., 1986; Wiche et al., 1986).

Two of the most well-characterized MAPs are the high molecular mass MAPs 1 and 2. With several exceptions (Weatherbee et al., 1982; Wiche et al., 1984), immunological studies have revealed that MAP-2 is almost exclusively found in neuronal tissue (Vallee, 1982; Caceres et al., 1984) or in cells thought to be derived from the neural crest (Stearns and Binder, 1987). By comparison, MAP-1 is found in neuronal tissue (Vallee et al., 1986; Bloom et al., 1984*a*,*b*,*c*) and in a number of mammalian cells as well (Bloom et al., 1984*a*; Vallee et al., 1986; Wiche et al., 1984; Asai et al., 1985; Sato et al., 1983). Lewis et al. (1986*a*,*b*) have confirmed these results using specific cDNA probes for MAP-2 and MAP-1. They found that MAP-2 was exclusively expressed in neuronal tissue whereas MAP-1 was expressed in a wide variety of cells and tissues.

In the studies of cultured cells, MAP-1 was not always found associated with microtubules, raising doubts as to whether it represented an authentic MAP in situ. Bloom et al. (1984*a*) and Wiche et al. (1984) found MAP-1 associated with interphase microtubules in several mammalian cells. In contrast, Sato et al. (1983) and Asai et al. (1985) found that MAP-1 antibodies did not label microtubules but instead stained intranuclear spots, centrosomes (Sato et al., 1983), and stress fibers (Asai et al., 1985). Clearly, further studies are needed to assess the subcellular distributions and properties of MAP-1 in nonneuronal cell systems.

In our studies of the distribution and related functional roles of MAPs and microtubules, we have used monoclonal antibodies specific for MAP-1 and -2. We have also attempted to identify a class of drugs which binds MAPs, disrupts microtubules, and inhibits the activities of MAPs in vivo. Initial studies have revealed that a novel anti-microtubule compound, estramustine, produces microtubule disassembly in situ (Stearns and Tew, 1985; Stearns et al., 1985). Likewise, an analogue of estramustine, estramustine phosphate, was found to inhibit brain microtubule assembly in vitro (Kanje et al., 1985; Wallin et al., 1985) as a result of its binding affinity for MAP-2 and tau proteins (Friden et al., 1987). Recently, we showed that estramustine-bound brain

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MAP-2 inhibited microtubule assembly and produced microtubule disassembly in vitro (Stearns and Tew, 1988).

In this study, we report the characterization of a "MAP-1-like" protein found in DU 145 human prostatic tumor cells. The purified protein promoted microtubule assembly in vitro and immunofluorescence studies showed that it was associated with microtubules in nondividing cells. Estramustine was found to bind purified MAP-1 and inhibit its associations with microtubules in vitro and in situ. The data demonstrate that estramustine is an anti-MAPs drug that can be used to study the role of MAPs in situ.

Materials and Methods

Microtubule Protein Purifications

DU 145 cells (passage 9) were grown to confluency in 60 150-mm^2 dishes using DME supplemented with 10% FCS. The cells were dissociated from the dishes with 0.08 M sodium citrate in PBS for 20–30 min and harvested by centrifugation at 5,000 rpm for 5 min. The cells (~4 g) were washed by centrifugation with 3 changes of PBS and resuspended in 10 ml of microtubule stabilization buffer (MSB)[1] at 4°C after the final wash. MSB consisted of 0.1 M Pipes, 5 mM $MgCl_2$, 5 mM EGTA, 0.1% aprotinin, 0.1% PMSF, 0.1% leupeptin, and 0.1% soybean trypsin inhibitor, pH 7.2. The mixture was homogenized with 20 up and down strokes in a 10-ml teflon to glass homogenizer (Wheaton Instruments Div., Millville, NJ) at 4°C and immediately centrifuged at 39,000 *g* for 30 min in a rotor (model SS34; Beckman Instruments Inc., Palo Alto, CA). The supernatant was removed with a pipette and 10 mM adenylimidodiphosphate (AMP-PNP) and 1.0 mM GTP added and the mixture incubated for 45 min at 37°C (step 1). The mixture was centrifuged at 39,000 *g* for 60 min to obtain a microtubule pellet (step 2). The pellet was resuspended in 5 ml MSB plus AMP-PNP and GTP at 4°C for 45 min, and recentrifuged at 39,000 *g* for 30 min (step 3). The supernatant was removed and steps 1–3 repeated to yield a partially purified microtubule mixture.

For purification of the MAPs, 10 μM taxol (Schiff et al., 1979) was added to the above microtubule mixture for 30 min. The mixture was made 0.2 M NaCl, vortexed at a setting of 6 for 1 min, and centrifuged at 55,000 rpm for 1 h in a swinging bucket rotor (model Ti 65; Beckman Instruments Inc.; step 4). At this step the samples were layered on a 1-ml cushion of MSB containing 15% sucrose, 10 mM AMP-PNP, and 10 μM taxol. The microtubule pellet (~0.3 g) was resuspended in 2 ml MSB containing 10 mM AMP-PNP, 10 μM taxol, and 0.2 M NaCl (step 5) and step 4 repeated. Steps 4 and 5 were repeated once more and the final microtubule pellet was resuspended in 1 ml MSB at 22°C containing 10 μM taxol, 0.4 M NaCl, and 10 mM ATP. The mixture was homogenized with 2 up-and-down strokes of a 5-ml homogenizer (teflon to glass; Wheaton Instruments Div.), and after 10 min centrifuged at 100,000 *g* for 1 h in a centrifuge (model TL100; Beckman Instruments Inc.). The supernatant containing the MAPs protein (~0.35 mg) was removed with a pipette and chromatographed with PBS on a 30 × 1.5-cm agarose column (A5m; Bio-Rad Laboratories, Cambridge, MA). The 330-kD protein (~0.12 mg) was eluted in 1-ml vol in fractions 24–29 and was found as a pure protein in fractions 24–26. Tubulin (6 S) was purified from 3× cycled microtubule proteins prepared according to standard methods of Murphy and Borisy (1975) and using phosphocellulose PC-11 (Whatman Inc., Clifton, NJ) chromatography (Weingarten et al., 1975). The protein concentration was determined using a kit from Bio-Rad by modified methods of Bradford (1976) and the protein aliquoted and frozen at −80°C. Samples were denatured for SDS-PAGE after the protocols of Laemmli (1970). For SDS-UREA-PAGE, 6 M urea was included in the denaturation buffer, the running buffer, and the gel. Proteins were subjected to electrophoresis on 8% acrylamide gels with a 4% stacking gel using a 40-mA current for 5–8 h. The purified 330-kD protein was subjected to electrophoresis on a 4–12% acrylamide gradient gel to assess purity. Gels were silver stained using a Bio-Rad kit and protocols of Morrissey (1981). Western blots were carried out by methods of Towbin et al. (1979) using MAP-1 antibodies (MI-AI), MAP-2 antibodies (AP-9, -13, and -14), and tubulin antibodies (27B) raised against bovine brain protein in the laboratory of Dr. Lionel Rebhun (University of Virginia, Charlottesville, Virginia).

1. *Abbreviations used in this paper*: AMP-PNP, adenylimidodiphosphate; MSB, microtubule stabilization buffer.

Microtubule Assembly Studies

Microtubule assembly studies were carried out using 6-S tubulin and the A5m column-purified 330-kD protein. The proteins were thawed and diluted to the appropriate concentrations in MSB containing 1 mM GTP and protease inhibitors (see above). Samples at 4°C were mixed together and put in cuvettes at 4°C before transferring to a DU7 spectrophotometer prewarmed to 37°C.

Electron Microscopy

Microtubules were centrifuged at 90,000 *g* for 2 h at 23°C to form a pellet and the pellet was fixed with 2% gluteraldehyde plus 0.1% tannic acid in 0.1 M cacodylate buffer for 15 min, washed 3 times with PBS, dehydrated with a graded series of ethanol (10–100%), and embedded, stained, and thin sectioned according to methods described by Stearns and Brown (1981). Sections were sputter coated with a thin layer of carbon using an evaporator (Denton Vacuum Inc., Cherry Hill, NJ) and examined in a Phillips 410 microscope.

Immunofluorescence Studies

DU 145 cells were cultured in DME containing 10% FCS using standard procedures (Ochs and Stearns, 1981). In brief, cells were allowed to attach on carbon-coated, glow-discharged glass coverslips or to carbon formvar-coated gold grids (200 mesh) for 1–2 d, cells were washed briefly in MSB for 1 min at 37°C and immersed in MSB containing 1% paraformaldehyde, 0.25% gluteraldehyde, 0.5% Triton X-100 for 4 min at 37°C. The fixed cytoskeletons were labeled by indirect immunofluorescence using IgG monoclonal antibodies specific for MAP-1, tubulin, and vimentin. Dilutions were prepared in PBS with 10% normal goat serum. The MAP-1 (MI-AI) antibody was used at a dilution of 1:200, the tubulin used at 1:100, and vimentin antibodies (Boehringer-Mannheim Biochemicals, Indianapolis, IN) used at a dilution of 1:4. Secondary antibodies made in rabbits, a rhodamine-conjugated anti-mouse IgG, and a fluorescein-conjugated anti-mouse IgG (Miles Scientific Div., Naperville, IL), were used at dilutions of 1:400. Cells were washed in three changes of PBS at pH 7.2 for immunolabeling by methods previously described (Ochs and Stearns, 1981). Double labeling studies were carried out using a modified antibody sandwich blocking technique of Kristofferson et al. (1986). After incubation with the first set of primary and secondary antibodies, cells were incubated with goat anti-rabbit IgG (Miles Scientific Div.) followed by rabbit anti-goat IgG. These incubations were followed by incubations with the second batch of primary and secondary antibodies. Control studies were carried out using preabsorbed primary antibody or secondary antibodies. The double labeling studies were also repeated using a polyclonal antitubulin IgG made in rabbits (courtesy of Janssen Pharmaceutica, Beerse, Belgium).

Studies of Permeabilized DU 145 Cells

DU 145 cells cultured on coverslips were gently lysed with 0.001% digitonin in MSB and incubated with 0.1 μM taxol for 20 min followed by solutions containing affinity-purified MAP-1 (MI-AI) and FITC-IgG-conjugated secondary antibodies at 0.1 μg/ml according to methods of Stearns and Ochs (1982). Cells were then exposed to different reagents (estramustine, estradiol, nor-nitrogen mustard) and fixed with 1% paraformaldehyde, 0.25% gluteraldehyde solutions in MSB for 5 min. The cells were washed with PBS, photographed, and stained with tubulin antibodies and RITC-IgG antibodies before rephotographing. Cells were examined by phase-contrast microscopy or epifluorescent microscopy using a 63× planapochromatic lens and Zeiss optics.

Drug

Estramustine was synthesized by A. B. Leo (Helsingborg, Sweden). The drug was stored at 4°C in the dark in benzene/ethanol (9:1). Just before use the benzene/ethanol was evaporated in a stream of nitrogen in a fume hood and the chemical purity of the drug determined by HPLC analysis. The drug was redissolved at 1 mg/ml in absolute ethanol, 10 μl of which was injected onto a 250 × 4.6-mm Cyano Spheri-5 column (Brownlee Labs, Santa Clara, CA). The mobile phase was heptane/isopropanol (92.5:7.5) at a flow rate of 1.5 ml/min, attenuation of 100 mAu, and detection wavelength of A_{230}.

HPLC analysis of the estramustine indicated it was >99% pure. HPLC also showed that solubilization in dimethylsulfoxide and aliquoting into a microtubule stabilizing buffer did not result in breakdown products after several hours indicating the drug was stable under these conditions.

Results

Double immunofluorescent studies with MAP-1 antibody revealed that a MAP-1-like protein colocalized with the interphase microtubules in DU 145 cells (Fig. 1, *a* and *b*). Double labeling with vimentin, tubulin, and MAP-1 antibodies indicated that microtubules and vimentin filaments often closely overlapped in the perinuclear region (Fig. 1, *c* and *d*) and that MAP-1 might possibly interact with both types of filaments (Fig. 1, *e* and *f*). Note that the MAP-1 patterns often ranged between the two extremes shown in Figs. 1, *b* and *d*, depending on the individual cell examined. Usually the frequency of coincident fiber staining by the three antibodies was ~80 percent and the remaining cells were too poorly stained to discern if discrete fiber patterns existed (see Fig. 1, *e* and *f*). MAP-2 antibodies raised against distinct epitopes (AP14, AP9, and AP13) failed to even faintly stain DU 145 cells (data not shown). Single antibody labeling studies consistently supported the double labeling data, ruling out possible errors arising for antibody binding nonspecifically in the studies. Also, background fluorescence from the aldehyde fixatives was minimal.

For further characterization of the MAP-1-like protein, the microtubules were partially purified from crude extracts of DU 145 cells. SDS-UREA-PAGE revealed that the isolated microtubule preparations contained tubulin, and a 330-kD protein plus numerous other proteins (Fig. 2). Western blots showed that MAP-1 antibodies raised against mammalian brain antigen (MI-AI) specifically bind the 330-kD protein (Fig. 3). MAP-2 antibodies raised against distinct MAP-2 epitopes (AP9, AP13, and AP14) did not bind any of the proteins. Comparative blots of bovine brain microtubules showed that MAP-1 antibody binds a 330 kD brain protein and that MAP-2 binds a 300-kD peptide indicating the antibodies were specific for their respective peptides (data not shown). Taken together the data indicate that a MAP-1-like protein is expressed in DU 145 cells. In contrast, MAP-2 does not appear to be expressed in DU 145 cells, since the particular MAP-2 antibodies used failed to detect an immunoreactive protein.

The 330-kD protein was purified for two purposes: (*a*) to characterize its ability to promote 6-S tubulin assembly and (*b*) to determine if estramustine can bind and inhibit its assembly promoting activities in vitro. Microtubules were isolated in an assembly buffer that contained 10 mM AMP-PNP, since addition of AMP-PNP to the buffer (as opposed to ATP) increased the amounts of the 330-kD protein present by an order of magnitude. During subsequent purification steps, the microtubules were assembled and stabilized with 10 μM taxol for 20 min before adding 10 mM AMP-PNP to the buffer. The microtubules were then centrifuged through a 15% sucrose cushion (three times) to obtain a microtubule fraction which consisted largely of tubulin and a prominent 330-kD protein (Fig. 2, lane *1*). Densitometric scans at 550 nm showed that the 330-kD protein represented ~10% of the total protein associated with the isolated microtubules. The MAPs were separated from the microtubules by resuspending the microtubules in buffer containing 10 μM taxol, 10 mM ATP, 0.4 M KCl, and 0.01% DTT. After centrifugation through a 15% sucrose gradient the microtubules were pelleted leaving the MAPs in the supernatant (Fig. 2, lane *2*). When the MAPs were eluted on an A5m agarose column, the 330-kD protein eluted as a pure protein in fractions 24–28 (Fig. 3). Silver-stained gels revealed very faint (diffuse) low molecular mass bands in these fractions. Western blots confirmed that the purified 330-kD peptide was MAP-1 and showed that MAP-2 could not be detected in the preparations (Fig. 3). The 330-kD protein usually migrated as a single band, although it may turn out to be a dimer (see Fig. 2).

Microtubule Assembly Studies: Inhibitory Effects of Estramustine

Turbidimetric (A_{350}) analysis revealed that the 330-kD protein stimulated assembly of 6-S tubulin purified from DU 145 cells (Fig. 4). The freshly purified 330-kD peptide (0.2 mg/ml) was mixed with 6-S tubulin (2 mg/ml) at 4°C and the mixture was allowed to warm to 37°C in the spectrophotometer. If 0.1 μM taxol was included in the mixture, an initial increase in turbidity occurred by 2 min and equilibrium was reached after ~7 min. In the other samples an increase in turbidity was recorded after ~7 min and equilibrium was reached by ~17 min. The addition of increasing amounts of estramustine (20–60 μM) to the mixture (at time zero) significantly diminished the rate of increase in turbidity and the final equilibrium reached in a dose-dependent manner. The turbidity at equilibrium was ~1/2 the maximum in samples containing 20 μM drug and near zero in samples containing 60 μM estramustine. By comparison, the addition of 60 μM estradiol plus 60 μM nor-nitrogen mustard to the protein mixtures did not affect the rate of change in turbidity or greatly diminish the final equilibrium achieved. The turbidity of tubulin (2 mg/ml) alone changed very little with or without the addition of 60 μM estramustine.

When 60 μM estramustine was added to a preparation at equilibrium, a dramatic decrease in turbidity was recorded over ~7 min. Reduced levels of 20 and 30 μM estramustine partially reduced the turbidity observed at equilibrium (data not shown). In contrast, when 60 μM estramustine was added to the taxol-stabilized microtubules it produced only a small decrease in turbidity after ~5 min, indicating the taxol microtubules were resistant to the drug effects. After centrifugation of these microtubules, SDS-UREA-PAGE showed that 60 μM estramustine had removed the 330-kD protein from the taxol-microtubules that accumulated in the pellets. Whereas in the absence of drug treatment, the 330-kD protein was associated with the microtubule pellet (Fig. 5).

Electron Microscopic Studies

The effects of estramustine on microtubule assembly were further examined by thin section electron microscopy. Fig. 6, *a* and *c* shows that microtubules made from the purified 330-kD protein and 6-S tubulin were coated with numerous filaments (*arrowheads*) which sometimes cross-linked adjacent microtubules. If these microtubules were stabilized with 0.1 μM taxol and then exposed to 60 μM estramustine for 20 min before fixation (and centrifugation), the microtubule surfaces were smooth in appearance (Fig. 6, *b* and *d*), indicating estramustine had removed the 330-kD protein from the microtubule surfaces. In agreement with these results, 60 μM estramustine almost completely inhibited microtubule formation (except for the occasional microtubule) when a mix-

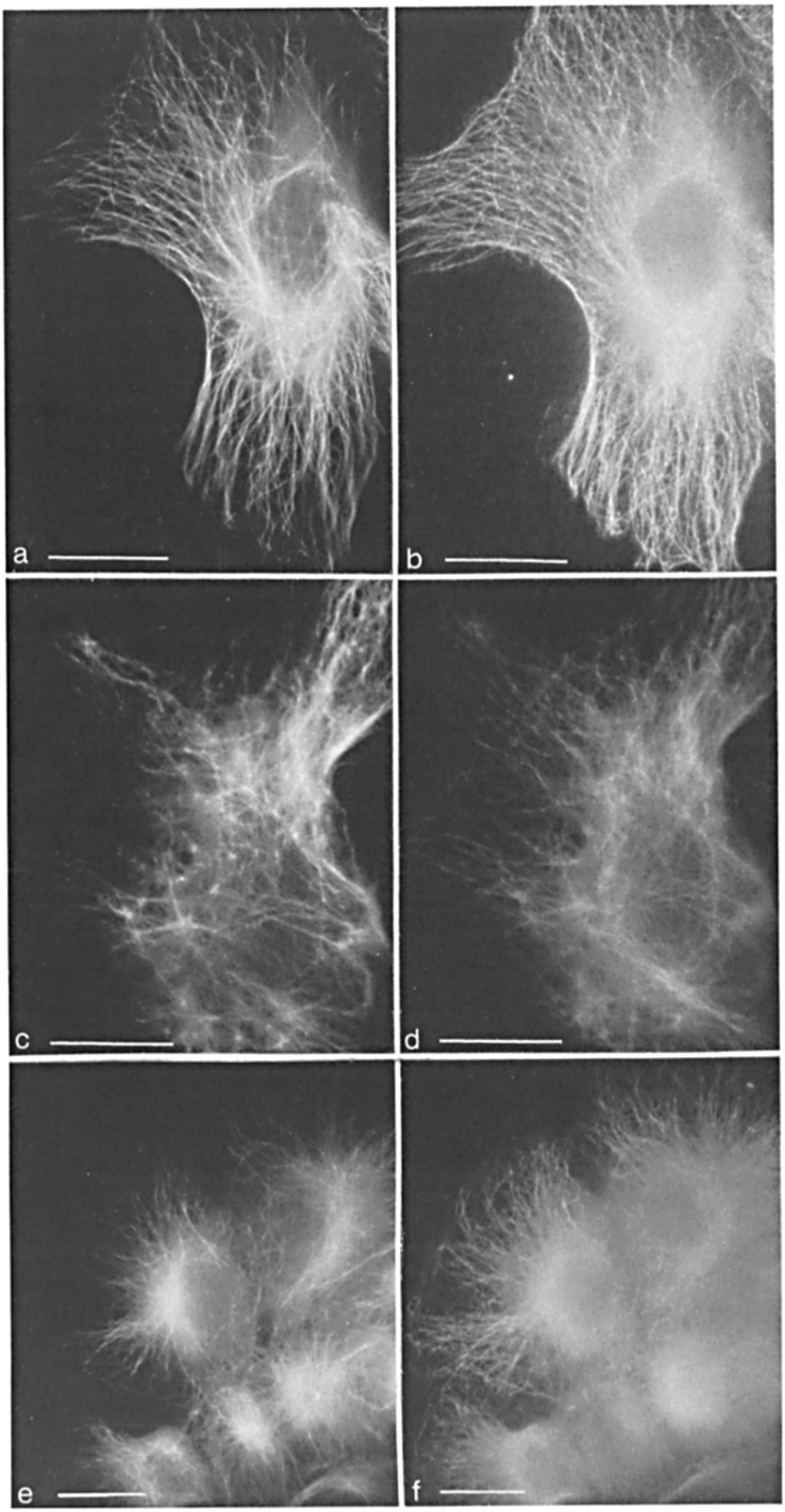

Figure 1. DU 145 cells double labeled with antibodies raised against tubulin, MAP-1, and vimentin. (*a* and *b*) Tubulin, MAP-1; (*c* and *d*) vimentin, MAP-1; (*e* and *f*) vimentin, tubulin. Demonstrates that MAP-1 and tubulin exhibit identical patterns; that vimentin filaments colocalize with MAP-1 and tubulin in the perinuclear region. Bar, 10 μm.

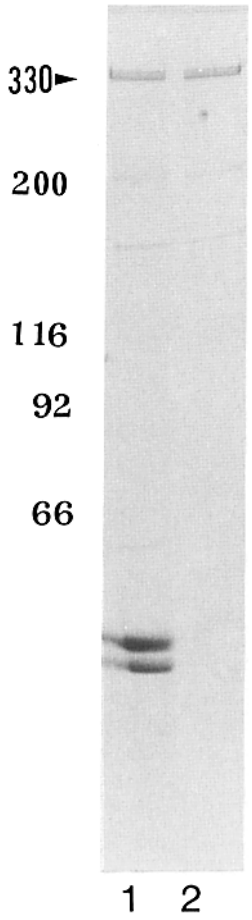

Figure 2. Silver-stained SDS-UREA-PAGE (8% gel) of microtubule proteins partially purified from DU 145 cells. (Lane *1*) Isolated microtubule fraction; (lane *2*) the 330-kD MAPs (*arrowhead*) removed with salt and ATP from the taxol microtubules. Numbers on the side indicate the molecular masses in kilodaltons.

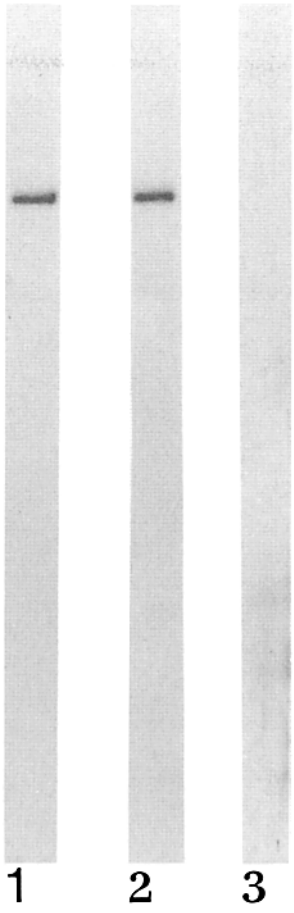

Figure 3. (Lane *1*) Amido black-stained nitrocellulose strip containing the 330-kD protein (3 μg). Western blots with (lane *2*) MAP-1 and (lane *3*) MAP-2 antibodies.

ture of the 330-kD (0.2 mg/ml) protein and 6-S tubulin (2 mg/ml) were incubated under assembly conditions in the absence of taxol. Instead numerous electron-dense aggregates formed as a result of drug treatment. Protofilaments or protofilament ribbons of partially formed microtubules were not found in any of the preparations (Fig. 6 *e*).

In Situ Effects of Estramustine

The effects of estamustine on microtubules were examined in intact and in digitonin permeabilized (i.e., partially lyzed) DU 145 cells. Fig. 7, *a–f* shows cells fixed and stained with tubulin antibody (27B) and MAP-1 antibody (MI-AI) after exposure to 60 μM estramustine for 5, 30, and 50 min. After 5-min exposure the microtubules were partially disassembled, apparently from their distal ends (Fig. 7, *a* and *b*). After 20 min, the only microtubules remaining were found at the cell center surrounding the perinuclear region (Fig. 7, *c* and *d*), and after 50 min exposure to drug, virtually no microtubules remained intact (Fig. 7, *e* and *f*). MAP-1 was found on the microtubules at all stages of their disassembly. However, at 50 min MAP-1 antibody labeled perinuclear filaments that remained behind after complete removal of the microtubules (Fig. 7 *f*). Exposure of the cells to high drug levels (120 μM estramustine for 50 min) failed to destroy these structures or to remove the MAP-1 antigen from their surface (Fig. 8, *a* and *b*). Double labeling studies with vimentin antibodies and MAP-1 antibodies showed that after complete disassembly of the microtubules, the MAP-1 antigen was bound to clumps of intermediate filaments (Fig. 8, *a* and *b*). Control studies using 60 μM estradiol and nor-nitrogen mustard failed to produce microtubule disassembly or disrupt the distribution of the 330-kD antigen (i.e., displace MAP-1 from the microtubule's surface).

As an alternate approach, we used unfixed, digitonin-permeabilized cells to test the effects of estramustine on the "taxol-stabilized" microtubules in cells. After permeabilization the microtubules were stabilized with 0.1 μM taxol for 20 min at 37°C. Immunofluorescent labeling with MAP-1 antibody and goat anti-mouse secondary antibodies (IgG-FITC) for 30 min at 37°C revealed that the 330-kD protein was associated with microtubules before drug treatment (Fig. 9 *a*). The cell was photographed for 10 s and then exposed to 60 μM estramustine for 30 min at 37°C before being rephotographed. The immunofluorescent image in Fig. 9 *b* revealed that estramustine treatment had removed most of the MAP-1 antibody IgG-FITC fluorescent signal from the microtubule surfaces. Some filamentous labeling was still observed in the perinuclear zone. After fixation, and relabeling with fresh MAP-1 antibody (and secondary antibody coupled to FITC) it was clear that the microtubules were indeed barren of any MAP-1, indicating the 330-kD protein was removed in response to estramustine. Subsequent labeling with tubulin antibody (27B) confirmed that the taxol-

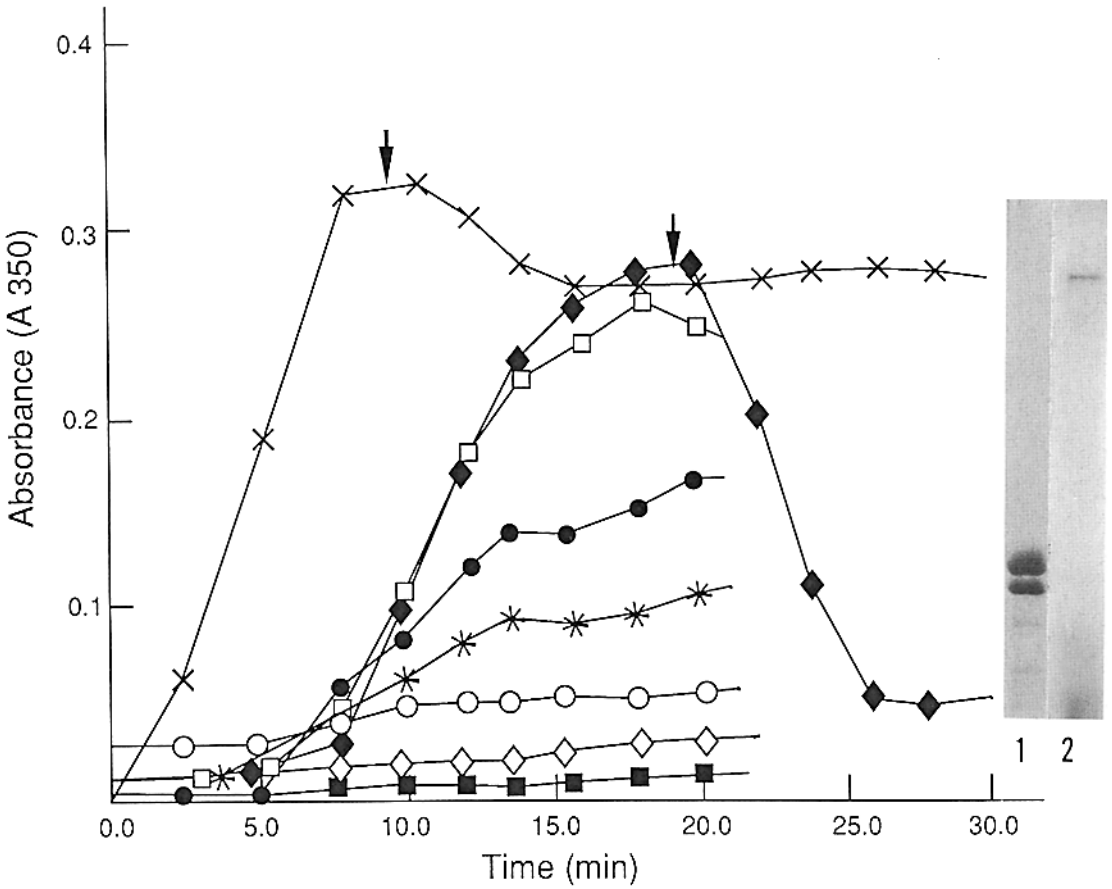

Figure 4. Turbidimetric (A_{350}) analysis of 6-S tubulin assembly. All the mixtures contained the 330-kD protein (0.2 mg/ml) and 6-S tubulin (2 mg/ml). The cuvettes contained (♦) protein only or protein plus: (X) 0.1 μM taxol; (□) 60 μM estradiol and 60 μM nor-nitrogen mustard; (○) 60 μM estramustine; (*) 30 μM estramustine; (●) 20 μM estramustine; (◇) 2 mg/ml tubulin only; (■) 2 mg/ml tubulin only plus 60 μM estramustine. The arrows indicate when 60 μM estramustine was added to the cuvettes containing protein only (♦) or protein plus taxol (X). (*Inset*) Silver-stained SDS-PAGE of the purified tubulin (lane *1*) and MAP-1 (lane *2*) used.

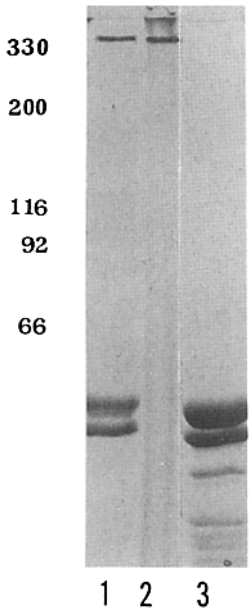

Figure 5. Silver-stained SDS-PAGE (8% gel) of taxol microtubules pelleted in the absence (lane *1*) and in the presence (lane *3*) of 60 μM estramustine. Lane *2* shows the supernatant from the preparation in lane *3*. Numbers indicate the molecular masses in kilodaltons.

stabilized microtubules had remained intact during the course of drug treatment (Fig. 9 *c*). Prolonged exposure to drug for 50 min did not reduce the extent of MAP-1 antibody staining in the perinuclear zone.

Photobleaching did not account for the reduction in MAP-1 antibody staining as the exposure times were limited to 10 s and relabeling with MAP-1 antibody did not enhance the MAP-1 signal. In control experiments with 60 μM estradiol and nor-nitrogen mustard the MAP-1 antibody staining was not reduced and the photographic exposure times used failed to bleach the signal (data not shown). Stereo high voltage immunogold electron microscopic studies of whole mount DU 145 cells supported the immunofluorescent observations reported in Fig. 9 (data not shown).

Discussion

In this paper, we have shown that a 330-kD MAP-1–like protein is present in DU 145 cells. Preliminary immunofluorescence studies and Western blot analysis with MAP-1A and MAP-1B monoclonal antibodies raised against brain antigens (courtesy of George Bloom, University of Texas, Dallas; Bloom et al., 1984*a*,*b*,*c*) have revealed that the 330-kD protein identified in DU 145 cells is probably MAP-1A (data not

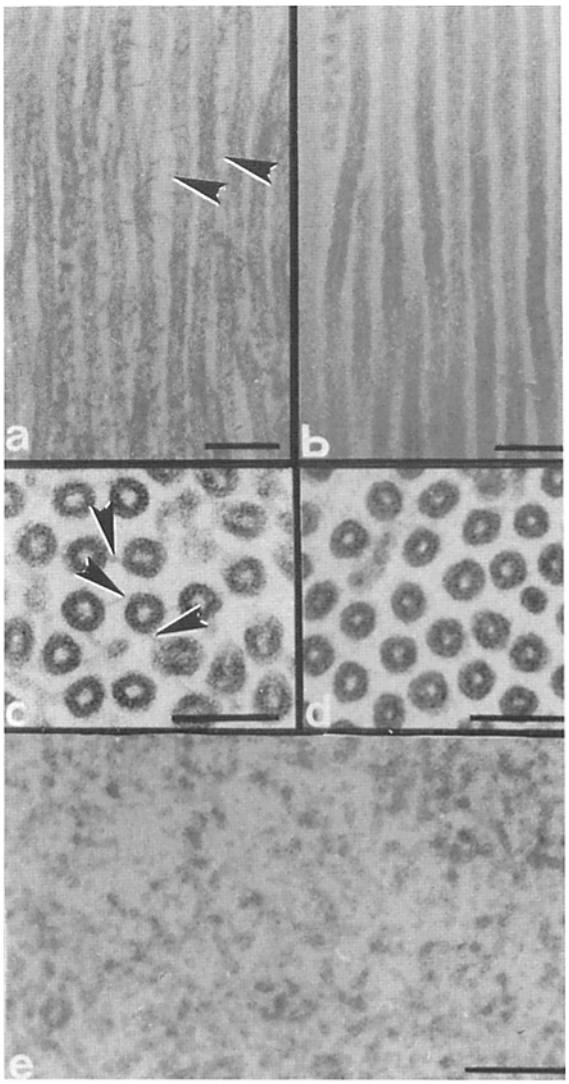

Figure 6. Thin sections of MAP-1 microtubules (*a* and *c*); of taxol-stabilized 6-S-microtubules incubated with 0.2 mg/ml MAP-1 and 60 μM estramustine for 20 min at 37°C (*b* and *d*); and of the aggregates formed when MAP-1 and 6-S tubulin were incubated under assembly conditions in the continual presence of 60 μM estramustine (*e*). The longitudinal (*a* and *b*) and cross section views (*c* and *d*) demonstrated that MAP-1 decorated the microtubule surfaces in the absence of drug (*a* and *c*, *arrowheads*) but not in the presence of drug (*b* and *d*). Bars, 0.1 μm.

shown). The 330-kD protein was found to coassemble with isolated microtubules and to promote 6-S tubulin assembly, indicating it acts like a true MAP in vitro and in situ. The properties of the 330-kD protein were examined with respect to the binding properties of a novel anti-microtubule drug, estramustine. Micromolar levels of estramustine were found to inhibit the assembly-promoting activities of the 330-kD protein and to produce disassembly of the microtubules assembled in the presence of this protein. Estramustine was partially effective at 20 μM levels and completely effective at 60 μM levels.

The apparent high levels of estramustine required for inhibition of MAP-1 binding to tubulin under physiological buffer conditions might arise from (*a*) limited drug solubility and binding efficiency under physiological conditions, (*b*) the kinetic effects of microtubule assembly–disassembly on drug binding, and (*c*) tubulin competition with estramustine for MAP-1 sites. With respect to the latter point, as much as 1/2 of the 330-kD molecule may associate with the microtubule surface (see Amos, 1977). Thus, accessibility of crucial segments of the MAP-1 molecule, the environmental effects and competitive binding by tubulin could drastically affect binding parameters.

Convincing evidence that estramustine functioned as an anti-MAP drug in situ was derived from immunofluorescent (and immunogold) labeling studies of DU 145 cells. Under conditions where taxol-stabilized microtubules were exposed to estramustine, the 330-kD protein was removed from the microtubule surfaces. This was a direct result of drug action related to unique properties of the synthetic compound (i.e., probably the carbamate-ester bond that links estradiol to nor-nitrogen mustard; Tew and Stearns, 1988), since the drug constituents, estradiol and nor-nitrogen mustard, did not bind the 330-kD protein or tubulin. The in situ observations were strongly supported by SDS-PAGE, turbidimetric, and electron microscopic studies of isolated microtubules which confirmed that estramustine had indeed removed the 330-kD protein from the surfaces of the taxol microtubules. We interpret the data in this manner for two reasons. Firstly, the "taxol-microtubules" appeared smooth after drug treatment in vitro or were no longer labeled with MAP-1 antibody in situ. Secondly, the drug prevented assembly of any intermediate forms of microtubules (i.e., rings or protofilamentous sheets). These intermediates would probably require specific MAP-tubulin binding at the concentrations of protein used. Thus, the electron-dense aggregates induced by estramustine were probably formed from MAP-1 drug complexes. Interestingly, this may mean that estramustine can form crystalline arrays in association with MAP-1, perhaps not dissimilar from vincristine-tubulin lattices.

In studies of intact cells we have previously reported that estramustine or dansylated estramustine (Stearns et al., 1985; Stearns and Tew, 1985; Stearns and Wang, 1987; Wang et al., 1987) diffused in and interfered with microtubules and normal cellular functions. For example, at reduced concentrations of ~30 μM, the drug inhibited intracellular transport (Stearns and Tew, 1985; Stearns and Wang, 1987) and partially reduced the population of microtubules. That is, partial disassembly of the microtubules was observed at the distal regions of the microtubules. Higher drug levels of 60–120 μM produced a rapid disassembly of the microtubules and a concomitant disruption of other cytomatrix components (Stearns et al., 1985; Wang et al., 1987). The cells stopped dividing and eventually died even at reduced levels of 10 μM estramustine (Stearns et al., 1985). The data presented here indicated that the principal target of estramustine in DU 145 cells is MAP-1 and that the manifested effects of estramustine on microtubules were a result of drug binding to the 330-kD protein. We cannot rule out the possibility that other MAPs present in DU 145 cells also might bind the drug. Several reports have shown that MAPs with molecular masses of 200–220-kD are present in cultured cell lines, including HeLa cells (Bulinski and Borisy, 1980; Weatherbee et al., 1980; Debrabander et al., 1981) and neuroblastoma cells (Olmsted and Lyon, 1981). Also, a 190-kD polypeptide has been found associated with microtubules of

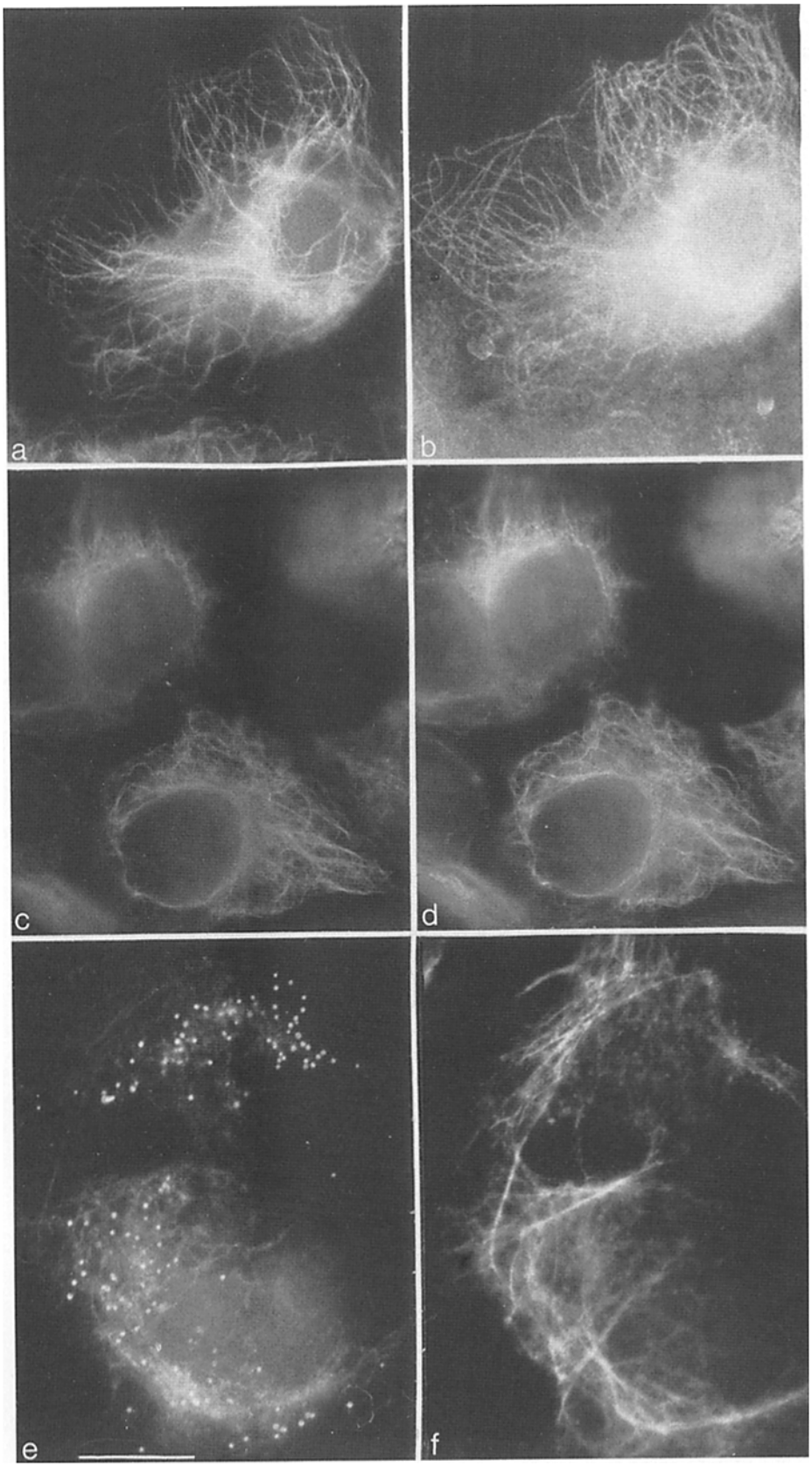

Figure 7. Immunofluorescence pictures of DU 145 double labeled with antibodies after exposure to 60 μM estramustine for (*a* and *b*) 5, (*c* and *d*) 30, and (*e* and *f*) 50 min. The cells were labeled with (*a*, *c*, and *e*) tubulin, and (*b*, *d*, and *f*) MAP-1 antibodies. Some MAP-1 was associated with vimentin filaments at 50 min (see Fig. 8). Bar, 10 μm.

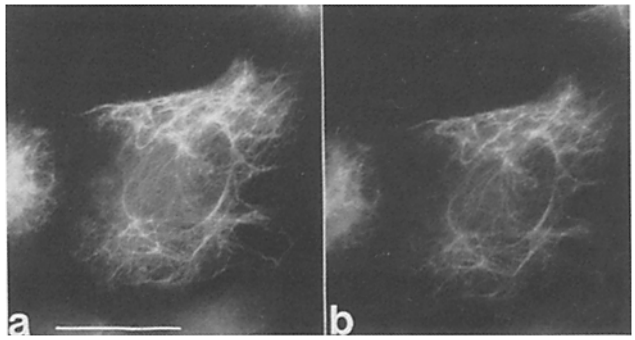

Figure 8. Immunofluorescence pictures of a DU 145 cell exposed to 120 μM estramustine for 50 min and double labeled with (*a*) MAP-1 and (*b*) vimentin antibodies. Bar, 10 μm.

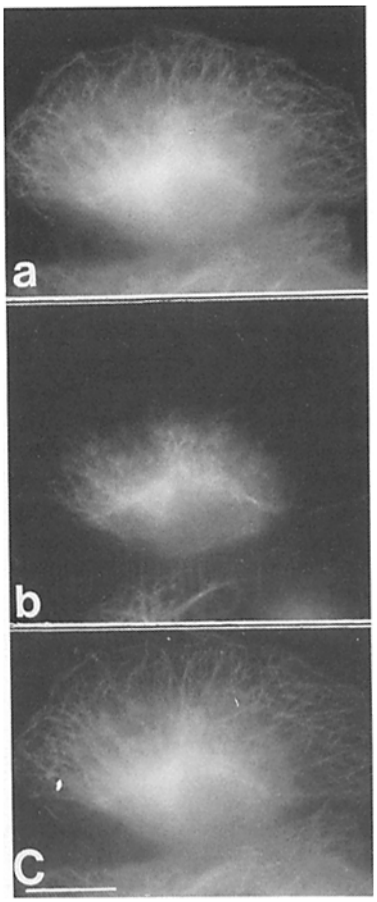

Figure 9. Immunofluorescence images demonstrating the effect of estramustine on MAP-1's distribution in DU 145 cells lysed with digitonin in MSB containing 0.1 μM taxol for 20 min. (*a*) MAP-1 associated with microtubules in the freshly lysed cell: (*b*) MAP-1 is only associated with perinuclear fibers after exposure of the same cell to 60 μM estramustine for 30 min at 37°C; (*c*) the microtubules are intact after 30 min exposure to the 60 μM estramustine. Antibody staining was carried out by adding MAP-1 antibody plus secondary antibody (1:400 dilution) to the lysis buffer for 30 min at 37°C followed by three washes with PBS. The cell was photographed (*a*) and exposed to 60 μM estramustine for 30 min at 37°C, then fixed and stained with tubulin antibody (1:100 dilution) and RITC IgG (1:400) for rephotographing (*b* and *c*). Bar, 10 μm.

cultured fibroblasts (Kotani et al., 1987). We have identified similar 205- and a 190-kD MAPs associated with microtubules isolated from DU 145 cells (unpublished data) using the assembly–disassembly protocols of Murphy and Borisy (1975). At this time, we have not determined if these MAPs can bind estramustine or if drug interaction with the 205- or 190-kD MAPs also affects microtubule stability.

Earlier studies in our laboratory revealed that estramustine binds pig brain MAP-1 and -2 to inhibit microtubule assembly and prevent MAP-1 and -2 binding 6S "taxol microtubules" (Stearns and Tew, 1988; Tew and Stearns, 1988). In addition, estramustine inhibited purified MAP-2's microtubule assembly–promoting activities. Initial kinetic binding studies showed that [^{3}H]estramustine binds purified MAP-2 with a K_d = 15 μM. B_{max} calculations revealed that ~20 molecules of estramustine bind each molecule of MAP-2. Likewise, preliminary binding studies showed saturation of estramustine binding sites on the purified DU 145 330-kD protein. Scatchard and nonlinear regression analysis gave a K_d of ~10 μM, and saturation occurred at 15 molecules of estramustine for each 330-kD molecule (data not shown). Under the physiological parameters for microtubule assembly, excess amounts of estramustine (~60 μM) were required to inhibit MAP-2's activities (e.g., at concentrations of 0.2 mg MAP-2 and 2 mg/ml 6S tubulin). This requirement for excess drug was identical to that observed for estramustine–MAP-1 interactions.

Continued studies with estramustine should help unravel critical problems in microtubule biology concerning how distinct microtubules are differentially assembled and disassembled. The process is complex as it involves MAPs, tubulin, and nucleotide interactions. Of considerable interest are the mechanisms and kinetics of tubulin addition (or loss) to the microtubule ends since MAPs are thought to assemble with tubulin and stabilize the elongating microtubules (Murphy et al., 1977). Likewise, the role(s) of MAPs in motility, cytoplasmic transport, secretion, and cell morphogenesis might be delineated using estramustine.

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