Interaction of Estramustine with Tubulin Isotypes†

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ABSTRACT: The interaction of the antimitotic agent estramustine with bovine microtubule proteins and purified tubulin was investigated. Direct photoaffinity labeling of microtubule protein with [^{14}C]-estramustine resulted in the labeling of both α- and β-tubulin, and this was inhibited with unlabeled estramustine in a dose-dependent manner. [^{14}C]Estramustine was incorporated into both the soluble and polymerized forms of tubulin. The affinity constant for estramustine binding to tubulin was determined by equilibrium dialysis to be 23 ± 5 mM. Estramustine did not affect [^{3}H]vinblastine binding, and vinblastine had no effect on direct labeling with [^{14}C]estramustine. Both rhizoxin and paclitaxel decreased the covalent labeling of tubulin with [^{14}C]estramustine in a dose-dependent fashion and were noncompetitive inhibitors of the binding of estramustine to tubulin. The binding of colchicine to tubulin was not inhibited by estramustine as detected by fluorescence and DEAE filter assays. The estramustine binding site on tubulin is therefore distinct from that of colchicine and vinblastine and may at least partially overlap with the binding site for paclitaxel. In both bovine brain microtubules and cytoskeletal proteins from human prostatic carcinoma cells, the incorporation of [^{14}C]estramustine into the β_{III} isotype of tubulin was found to occur with a reduced efficiency compared to that of the other β-tubulin isotypes and α-tubulin. Since this isotype is overexpressed in estramustine resistant human prostate carcinoma cells, these results indicate that β_{III}-tubulin may play a role in the response to the effects of estramustine.

Estramustine (EM) is an effective agent in the treatment of advanced prostatic carcinoma especially when used in combination with other antimitotic agents (Hudes et al., 1992, 1995; Seidman et al., 1992; Amato et al., 1992). EM consists of an estradiol molecule linked to nornitrogen mustard through a carbamate bond. The drug was synthesized over two decades ago on the basis of the theory that the estrogen carrier would deliver a cytotoxic mustard species to tumor cells with the requisite steroid receptors (Fex et al., 1967). The stability of the carbamate bond of EM allows for its long clinical half-life *in vivo* (Gunnarsson et al., 1981, 1984). Several *in vitro* studies have demonstrated that the drug had pronounced cytotoxicity independent of any hormonal or alkylating activities [for a review, see Tew (1984)]. In human prostatic carcinoma cells, EM inhibits cell growth and clonogenic survival and induces mitotic arrest (Hartley-Asp, 1984; Tew & Hartley-Asp, 1984). Although the drug has demonstrated antimicrotubule effects, the specific mechanism of action has not yet been elucidated. While previous studies utilizing radiolabeled EM and estramustine phosphate have documented the affinity of the drug for microtubule-associated proteins (MAPs; Friden et al., 1991; Stearns & Tew, 1988), it has also been shown that EM can inhibit the polymerization of purified tubulin (Dahllöf et al., 1993). Because EM in combination with other antimicrotubule agents can have synergistic cytotoxicity either *in vitro* (Mareel et al., 1988; Speicher et al., 1992) or in patients (Hudes et al., 1992, 1995), to define the EM binding site on tubulin relative to other known tubulin binding drugs was of interest.

To facilitate more precise characterization of protein target(s) of EM, a photoaffinity analog of EM was previously synthesized and reacted with microtubule proteins (Speicher et al., 1994). The photoaffinity substituent of the EM analog provided a highly activatable aryl azide group and an ^{125}I label for improved detection. The analog maintained the cytotoxic properties of EM, and cultured tumor cells resistant to EM exhibited cross resistance to the analog. In DU145 cells, the analog covalently labeled both tubulin and MAP4, and this labeling was specifically inhibited with unlabeled EM (Speicher et al., 1994). There are some disadvantages to the use of photoaffinity analogs for the identification and characterization of ligand binding sites on a protein. Photoaffinity analogs generally exhibit higher levels of nonspecific labeling, and the analog may have a sufficiently different structure with respect to the parent drug that it may have a different mode of binding to, or a lower affinity for, its target protein(s). For example, previous studies with photoaffinity analogs of colchicine have shown that the subunit of the tubulin dimer labeled depends on the presence or absence of a spacer arm between the aryl azide group and the parent drug and the spacer length (Williams et al., 1985; Floyd et al., 1989). Since direct photoaffinity labeling is advantageous because it covalently cross-links the natural ligand to its binding site, we probed the EM binding site on microtubule proteins and tubulin by direct cross-linking with [^{14}C]-EM. Since tubulin was prominently labeled and it has distinct binding sites for vinca alkaloids, colchicine, and the nucleotide GTP, competitive photolabeling and direct binding studies with [^{14}C]EM and these ligands were performed. The results give some insight into the relative location of the EM binding site on tubulin.

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Both α- and β-tubulins are encoded by several genes each, and multiple isotypes are expressed in cells (Sullivan, 1988). The individual isotypes are highly conserved across species and differ from each other in amino acid sequence predominantly at the carboxy terminus. Several *in vitro* studies have shown that β-tubulin isotype composition affects microtubule dynamics, stability, and sensitivity to antimicrotubule agents (Khan & Luduena, 1995; Laferriere & Brown, 1995; Banerjee & Luduena, 1992). We have demonstrated previously that EM resistant cells are not cross resistant to other antimicrotubule drugs which are part of the multidrug resistant (MDR) phenotype (Speicher et al., 1991, 1994). The mechanism(s) by which resistance is expressed has yet to be determined. However, the fact that resistance is accompanied by an increased expression of the β_{III} isotype of tubulin (Ranganathan et al., 1996) could be construed as causal, providing that EM avidity for this isotype is diminished. The reduced affinity of EM for the β_{III} isotype of tubulin from either bovine brain or DU145 human prostatic carcinoma cells is reported in this paper.

EXPERIMENTAL PROCEDURES

Materials. Vinblastine, colchicine, podophyllotoxin, and antibodies against α-tubulin were obtained from Sigma Chemical Co. (St. Louis, MO). Rhizoxin was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. [^{3}H]Colchicine (6.7 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE), while [^{3}H]vinblastine (9 Ci/mmol) and [^{3}H]GTP (11 Ci/mmol) were from Moravek Biochemicals, Inc. (Brea, CA). [^{14}C]EM (2.5 mCi/mmol) was obtained from Pharmacia (Lund, Sweden) and was prepared from [^{14}C]phosgene (Fex, 1967). Radiochemical purity was 99.0% and was determined with reverse phase HPLC. Chemical purity was >95% according to silica gel thin layer chromatography with a fluorescence indicator. The ^{14}C label was present on C (21−24) of estramustine. Monoclonal antibodies to β_{II}-, β_{III}-, and β_{IV}-tubulin isotypes were obtained from Biogenex (San Ramon, CA). The monoclonal α-tubulin antibody was obtained from ICN (Costa Mesa, CA). Equilibrium dialysis was performed with a 1 mL equilibrium dialysis chamber and 6000 MW cutoff dialysis membrane from Fisher (Pittsburgh, PA).

Microtubule Protein Purification. Bovine brain microtubules were prepared according to the pH- and temperature-dependent method of Tiwari and Suprenant (1993). The twice-cycled microtubule protein pellet was resuspended in PEM [0.1 M PIPES (pH 6.6), 1 mM EGTA , 0.1 mM GTP, and 1 mM $MgSO_4$]. DEAE-Sephadex chromatography was performed according to the method of Vallee (1982), to separate tubulin from the MAPs.

DEAE-Cellulose Filter Assay for [^{3}H]Colchicine Binding. The binding of [^{3}H]colchicine to tubulin was followed on DEAE-cellulose filters (Borisy, 1972). The samples contained 0.1 mg/mL tubulin in colchicine binding buffer [1.0 M monosodium glutamate (pH 6.6), 0.1 mM GTP, 1 mM $MgCl_2$, and 5% DMSO], 5 mM [^{3}H]colchicine, and various concentrations of drugs. After a 20 min incubation at 37 °C, aliquots of 50 μL were applied to three layers of DEAE filter paper, and the filters were washed with buffer (10 mL/filter, 5 min/wash), added to scintillation fluid, and then counted.

Colchicine Fluorescence Assay. The fluorescence of colchicine upon binding to tubulin was measured with excitation at 362 nm and emission at 435 nm (Bhattacharyya & Wolff, 1974). Reaction mixtures contained 1.1 mg/mL tubulin in colchicine binding buffer and were preincubated with estramustine or podophyllotoxin for 5 min at 37 °C. Colchicine (5 μM) was added, and fluorescence of the sample was measured after incubation for 30 min at 37 °C.

Centrifugal Gel Filtration Assay. The binding of [^{3}H]-vinblastine, [^{14}C]EM, and [^{3}H]GTP to tubulin was measured with 1 mL columns of Sephadex G-25 (superfine). Reaction mixtures [0.35 mL containing tubulin (0.11 mg/mL), 0.1 M MES (pH 7.0), 0.5 mM $MgCl_2$, and 5% DMSO] were preincubated with or without competing drug for 5 min at 22 °C prior to the addition of [^{3}H]vinblastine (5 μM, 0.1 μCi), [^{3}H]GTP (5 μM, 0.2 μCi), or various concentrations of [^{14}C]EM (5−100 μM, 0.004−0.090 μCi). After incubation for 20 min at 22 °C, triplicate aliquots of 0.1 mL were applied to prespun 1 mL DEAE-Sephadex columns. Aliquots of the eluent obtained after centrifugation (5000*g* for 5 min) were taken for scintillation counting and for protein determination. At least three separate experiments were performed for each K_i determination, and the average K_i value ± SEM is reported.

Photoaffinity Labeling of Tubulin with [^{14}C]EM. For the direct labeling of microtubule proteins with [^{14}C]EM, microtubule proteins or tubulin (5 μM in tubulin in 0.1 M MES, 0.5 mM $MgCl_2$, and 5% DMSO at pH 7.0) was preincubated with various concentrations of drug for 5 min before the addition of 10 μM [^{14}C]EM. After incubation for 20 min at 22 °C, the proteins were irradiated for 10 min in a Stratagene UV linker with a 254 nm filter and then separated on an 8% SDS−PAGE gel. The gels were then dried and exposed to a [^{3}H/^{14}C] sensitive phosphorimaging screen for 24−48 h.

Specific Activity Determination of Labeled Tubulin. For the paclitaxel centrifugation experiments (Figure 4), gels of the labeled tubulin were stained with Coomassie blue and the relative amounts of tubulin in each lane were evaluated with the Gelscan XL program (Pharmacia). The relative amounts of radioactivity associated with each tubulin band were quantitated with the MacBas version 2.2 phosphorimager. Specific activity was determined by dividing the radioactivity by the relative amount of protein in each band.

Alkylation of Microtubule Proteins. Samples (0.1 mL) were labeled with [^{14}C]EM as described above and then diluted to 0.8 mL to bring the final concentrations: 8 M urea, 0.12 M mercaptoethanol, 0.1% EDTA, and 0.35 M Tris-HCl (pH 8.8). Sodium iodoacetate was then added (1.2 mM), and the samples were stored at 22 °C for 1 h (Crestfield et al., 1963). Samples were diluted to 2 mL with deionized water and then centrifugally concentrated with Centricon-10 concentrators. The protein concentrations of the samples were determined by the Bradford method.

Cytoskeletal Protein Preparation and Labeling. Cytoskeletal proteins were isolated from DU145 cells according to the method of Thrower et al. (1991). Cytoskeletal proteins (250 μg) in 0.5 mL of buffer (0.1 M MES, 0.5 mM $MgCl_2$, and 1 mM EGTA at pH 7.0) were incubated with [^{14}C]EM (30 μM) for 10 min at 37 °C, irradiated for 15 min, and then alkylated as described above. The samples (50 μg of protein) were then loaded onto a 10% SDS−PAGE gel, separated, and then transferred to a PVDF membrane for probing with tubulin antibodies.

Two-Dimensional SDS−PAGE. Isoelectric focusing of irradiated protein samples was performed according to the method of O'Farrell (1975). The second dimension was

carried out on 8% polyacrylamide gels (Laemmli, 1970) followed by transfer of proteins to a PVDF membrane or silver staining of the gels. After exposure to an imaging screen for 24−158 h, the blots were then probed with antibodies to α-, β_{II}-, β_{III}-, or β_{IV}-tubulin and developed after the addition of each primary antibody to localize the specific tubulin proteins. The relative amounts of protein associated with the spots for α-, β_{III}-, and the other β-tubulin isotypes were determined by scanning of the stained gels with a laser densitometer. The specific activity of each of the isotypes was determined by quantitating the radioactivity and correcting for the amount of protein present in each spot.

Equilibrium Dialysis with [^{14}C]EM and Tubulin. Tubulin (0.5−1.0 mg/mL in 0.1 M MES, 0.5 mM $MgCl_2$, 0.1 mM GTP, and 3% DMSO) was combined with various concentrations of [^{14}C]EM (5−75 μM) and dialyzed at 4 °C against buffer with the same [^{14}C]EM concentrations. Aliquots from the buffer and tubulin chambers were removed after 2 h for scintillation counting and protein determination.

RESULTS

Direct Photolabeling of Tubulin with [^{14}C]EM and Direct Binding of [^{14}C]EM to Tubulin. Incubation of tubulin (10 μM) with [^{14}C]EM and exposure to UV light (254 nm) for 10 min followed by SDS−PAGE revealed that covalent bond formation between tubulin and [^{14}C]EM occurred (Figure 1a). This labeling was inhibited with increasing concentrations of unlabeled EM. Under the conditions employed, it was not possible to inhibit completely the covalent incorporation with 100 μM unlabeled EM. The residual labeling observed in the presence of excess unlabeled EM presumably reflects nonspecific binding. Irradiation of tubulin results in a small amount of cross-linking of tubulin subunits (Speicher et al., 1994) which forms a protein band equivalent to 110 kDa on the gels. The covalent dimer of tubulin was labeled with [^{14}C]EM, and this was also competitively inhibited with EM.

The interaction of [^{14}C]EM with tubulin was characterized by equilibrium dialysis with several concentrations of [^{14}C]-EM. The resulting Scatchard plot indicates the presence of two classes of EM binding sites on tubulin. There is a high-affinity site ($K_d = 23 \pm 5$ μM) and a low-affinity site ($K_d = 210 \pm 35$ μM).

Interaction of the EM and Colchicine Binding Sites. In order to investigate the interaction between EM and the colchicine binding site, several different experiments were performed. Purified tubulin (5 μM) was preincubated with podophyllotoxin, a compound with high affinity for the colchicine site. After the addition of [^{14}C]EM (10 μM), the samples were further incubated, irradiated with UV light, and then subjected to SDS−PAGE. Podophyllotoxin had very little effect on the covalent labeling of tubulin with [^{14}C]-EM (Figure 2a).

The effect of EM on the binding of colchicine to tubulin was determined with a tubulin-induced colchicine fluorescence assay and a DEAE filter binding assay. The binding of colchicine to tubulin was not inhibited by EM concentrations of up to 100 μM in both assays (Figure 2b,c). Podophyllotoxin and unlabeled colchicine were used as a positive control for competitive inhibition of colchicine binding. Estradiol and 2-methoxyestradiol have tubulin-depolymerizing effects and have been shown to interact with the colchicine binding site (D'Amato et al., 1994). Although these compounds have a structure very similiar to that of

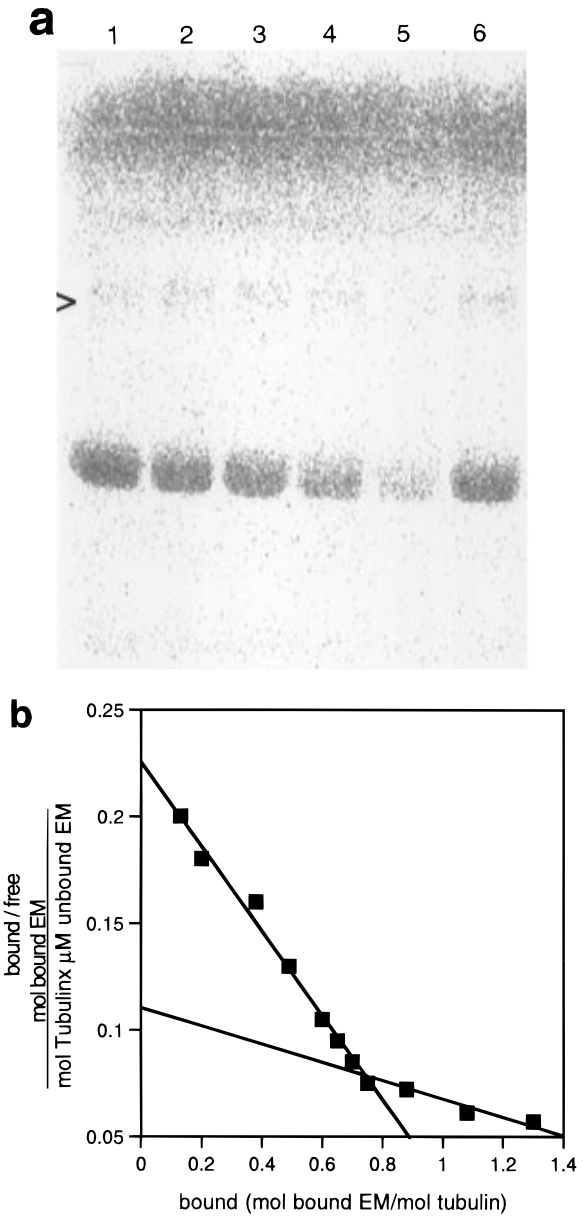

FIGURE 1: (a) Covalent labeling of tubulin with [^{14}C]EM after ultraviolet irradiation. MTPs were incubated with different concentrations of unlabeled estramustine for 5 min prior to the addition of [^{14}C]EM (10 μM). After 20 min at 22 °C, the samples were irradiated and then loaded on an 8% acrylamide gel. The dried gel was then exposed to an imaging screen for 48 h. Unlabeled estramustine concentrations were as follows: lane 1, 0 μM; lane 2, 3 μM; lane 3, 10 μM; lane 4, 30 μM; lane 5, 100 μM; and lane 6, 0 μM. The position of the covalently linked tubulin dimer is marked with an arrowhead. (b) Scatchard plot of the binding of EM to tubulin determined by equilibrium dialysis. The binding was determined as described in Experimental Procedures in the presence of various concentrations of EM.

EM, they showed no competitive inhibition of tubulin labeling with [^{14}C]EM (data not shown). These data suggest that EM does not interact with the colchicine binding site on tubulin.

EM and the Interaction of Paclitaxel with Tubulin. Paclitaxel is a novel drug that binds to tubulin and enhances microtubule polymerization. The presence of paclitaxel caused a dose-dependent reduction in the photoincorporation of [^{14}C]EM into tubulin (Figure 3a). Paclitaxel and EM were similar in the extent to which they inhibited the binding of [^{14}C]EM to tubulin (Figures 1a and 3a). The direct interpretation of these results would be that paclitaxel inhibits

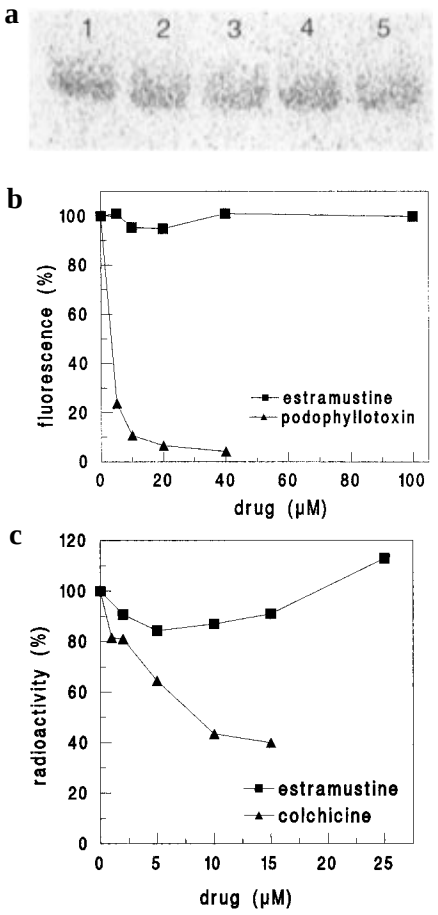

FIGURE 2: Lack of interaction between the binding sites for EM and colchicine or the colchicine analog podophyllotoxin on tubulin. (a) The effect of podophyllotoxin on the labeling of tubulin with [^{14}C]EM. The experiment was performed as described in Figure 1a, using podophyllotoxin instead of unlabeled estramustine. Podophyllotoxin concentrations were as follows: lane 1, 0 μM; lane 2, 3 μM; lane 3, 10 μM; lane 4, 30 μM; and lane 5, 50 μM. (b) The effect of EM and podophyllotoxin on tubulin-induced colchicine fluorescence. Purified tubulin samples were incubated with various concentrations of EM or podophyllotoxin for 5 min prior to the addition of colchicine. The fluorescence of the sample was measured after 30 min of incubation at 37 °C. EM had no effect on colchicine binding, whereas podophyllotoxin effectively inhibited colchicine binding. (c) Competitive inhibition of [^{3}H]-colchicine binding by unlabeled colchicine but not estramustine. Tubulin was incubated with [^{3}H]colchicine and various concentrations of drugs and applied to DEAE filters to separate bound from unbound drug as described in Experimental Procedures.

the binding of EM to tubulin. However, there was a possibility that the effect observed was actually due to the polymerization-enhancing properties of paclitaxel and not due to direct inhibition of [^{14}C]EM binding. Paclitaxel may have inhibited the interaction of [^{14}C]EM with tubulin through a reduction in the concentration of the tubulin monomer. This would require that EM possess a higher affinity for the monomeric than for the polymeric form of tubulin. In order to address this issue, microtubule proteins were incubated with [^{14}C]EM under conditions that promote microtubule polymerization, subjected to UV irradiation, and then centrifuged at 37 °C to separate the polymerized tubulin from the soluble monomeric tubulin. From the resulting gel (Figure 3b), it was apparent that [^{14}C]EM was incorporated into the tubulin found in both the microtubule pellet and the supernatant, when the polymerization was promoted with DMSO. The specific activities of [^{14}C]EM incorporation into the soluble and polymerized tubulin fractions were similar

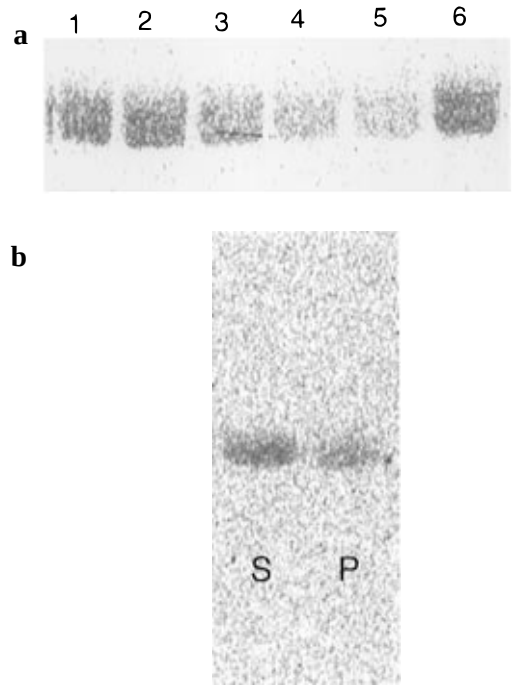

FIGURE 3: Effect of paclitaxel on the interaction of tubulin with [^{14}C]EM. (a) Inhibition of the photolabeling of tubulin with [^{14}C]-EM by paclitaxel. The experiment was performed as described in Figure 1a, using paclitaxel in place of unlabeled estramustine. Paclitaxel concentrations were as follows: lane 1, 0 μM; lane 2, 3 μM; lane 3, 10 μM; lane 4, 30 μM; lane 5, 100 μM; and lane 6, 0 μM. b) Labeling of both the soluble and polymerized forms of tubulin with [^{14}C]EM. Microtubule proteins were incubated under conditions that promote polymerization in the presence of [^{14}C]-EM and DMSO and then centrifuged to pellet the microtubules. The pellet and supernatant fractions were then loaded on an 8% SDS−polyacrylamide gel: S, supernatant fraction; and P, microtubule pellet.

(17.2 and 18.6 arbitrary units, respectively). Therefore, the inhibition of tubulin labeling with [^{14}C]EM observed in the presence of paclitaxel was not simply due to a reduction in the concentration of the soluble tubulin monomer.

The mode of paclitaxel inhibition of the binding of EM to tubulin was investigated with a centrifugal gel filtration assay. The data were analyzed according to Dixon (1979; Figure 4a) and Hanes (1932; Figure 4b). From Figure 4a, the K_i for paclitaxel inhibition of EM binding to tubulin was determined to be approximately 17 ± 5 μM. Since the lines of the Hanes plot intersect at the abscissa (Figure 4b), the inhibition was noncompetitive. Parallel lines were not observed on the Hanes plot, indicating that the inhibition was not competitive. Therefore, the EM binding site on tubulin is separate from that of paclitaxel, and the binding of paclitaxel to tubulin changes the affinity of tubulin for EM.

The Vinca Alkaloid Site and EM. The vinca alkaloids are a group of antimicrotubule agents known to bind to tubulin at a site distinct from that of colchicine. The effect of EM on the binding of [^{3}H]vinblastine to tubulin is presented in Figure 5a. The presence of EM at concentrations as high as 50 μM did not reduce the level of [^{3}H]vinblastine binding, whereas binding was competitively inhibited with unlabeled vinblastine. In addition, there was little effect of vinblastine on the covalent labeling of tubulin with [^{14}C]EM (quantitated in Figure 5b). These results would suggest that EM and vinblastine have separate and noninteracting binding sites on tubulin.

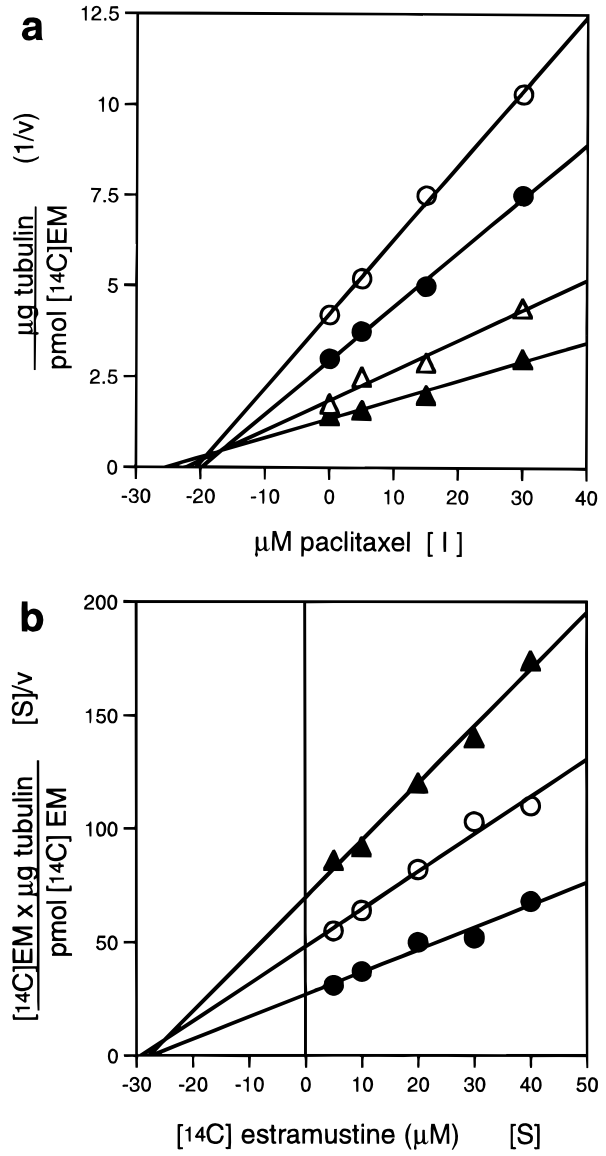

FIGURE 4: Effect of paclitaxel on the binding of [^{14}C]EM to tubulin. The amount of [^{14}C]EM bound to tubulin was determined with a centrifugal gel filtration assay as described in Experimental Procedures. (a) Dixon plot for the inhibition of the binding of [^{14}C]-EM to tubulin by paclitaxel. The following concentrations of [^{14}C]-EM were used: (○) 10 μM, (●) 20 μM, (△) 40 μM, and (▲) 70 μM. (b) Hanes analysis of the binding of [^{14}C]EM to tubulin in the presence of paclitaxel. The following concentrations of paclitaxel were used: (●) 0 μM, (○) 10 μM, and (▲) 20 mM.

Rhizoxin is a unique compound that interacts with the vinca alkaloid domain of tubulin. It reportedly does not act as a competitive inhibitor of [^{3}H]vinblastine binding (Takahashi et al., 1987) but was found to competitively inhibit the binding of [^{3}H]vincristine to tubulin (Bai et al., 1990). In the present study, rhizoxin was found to inhibit the covalent labeling of [^{14}C]EM with tubulin (Figure 5b). Rhizoxin was more effective than vincristine in reducing the incorporation of [^{14}C]EM into tubulin (Figure 5b). The ability of rhizoxin to inhibit the interaction of [^{14}C]EM with tubulin was further characterized with a centrifugal gel filtration assay. The Dixon plot for this analysis is presented in Figure 5c. The K_i for rhizoxin was determined to be 15 $\pm$ 4 μM. Hanes analysis of rhizoxin inhibition (Figure 5d) indicates that the inhibition was noncompetitive. Therefore, rhizoxin and EM have separate binding sites on tubulin.

GTP−GDP Exchange and the Effects of EM. The vinca alkaloid site is thought to be close to the exchangeable nucleotide site on tubulin, since some compounds (including rhizoxin) that bind to this domain inhibit the exchange of GDP for GTP (Bai et al., 1990). The effect of EM on the exchange of GDP for [^{3}H]GTP is shown in Figure 6a. The interaction of EM with tubulin differs from that of rhizoxin in that there is no inhibition of GDP−GTP exchange at high EM concentrations. It is also apparent that GTP had little effect on the labeling of tubulin with [^{14}C]EM at concentrations of up to 500 μM (Figure 6b). Therefore, the GTP- and GDP-bound forms of tubulin exhibit the same affinity for EM, and EM is unlike rhizoxin in that it does not inhibit GTP−GDP exchange on tubulin.

Interaction of [^{14}C]EM with the Different Isotypes of β-Tubulin. The extent to which the β_{III} isotype was labeled with [^{14}C]EM was determined and compared to that observed with the rest of the isotypes of β-tubulin found in bovine brain microtubules. In order to enhance the separation of β_{III}-tubulin from the other isotypes and from α-tubulin during two-dimensional SDS−PAGE, the proteins were reduced and carboxymethylated after covalent labeling with [^{14}C]EM. In panel 1 of Figure 7, β_{III}-tubulin is shown as being clearly separated from the other β-tubulin isotypes and from α-tubulin in a Western blot. Complete separation of β_{III}-tubulin from the other β-tubulin isotypes was consistently observed in at least five different blots. The radioactive image of the blot is presented in panel 2 of Figure 7. Duplicate samples were run and stained with Coomassie blue to give approximate quantitation of the amount of protein associated with the distinct tubulin spots (panel 3 of Figure 7). The efficiency of labeling with [^{14}C]EM was determined by quantitating the radioactivity associated with each spot in at least three different samples and correcting for the amount of protein (Table 1). When compared to the other β-tubulin isotypes, β_{III}-tubulin incorporated [^{14}C]EM with a 3-fold-reduced specific activity, and presumably, this reflects a lower affinity of this isotype for EM. This difference was more apparent with cytoskeletal proteins from DU145 wild type and EM resistant cells (Figure 8), where no detectable labeling of β_{III}-tubulin was observed.

DISCUSSION

Direct photolabeling was employed in order to investigate the interaction of EM with tubulin. The binding site appears to encompass both α- and β-tubulin, since both subunits were covalently labeled with [^{14}C]EM. This result is in contrast to the reports on the direct photolabeling of tubulin with other compounds that regulate the polymerization of tubulin. The β-subunit of tubulin was preferentially labeled by [^{3}H]-colchicine (Wolff et al., 1991), [^{3}H]GTP (Nath et al., 1985), and [^{3}H]paclitaxel (Rao et al., 1992). However, direct photolabeling of tubulin with [^{3}H]vinblastine (Wolff et al., 1991) or with a photoaffinity analog of vinblastine (Safa et al., 1987) resulted in labeling of both α- and β-tubulin, with more of the label being associated with α-tubulin in both cases. In the present study, the binding experiments with [^{3}H]vinblastine indicate that EM and vinblastine have distinct binding sites. Rhizoxin binds to at least part of the vinca alkaloid domain and was found to inhibit the interaction of EM with tubulin in a noncompetitive manner. Therefore, the binding of rhizoxin to tubulin causes a change in a separate EM binding site which reduces the affinity of tubulin for EM. However, EM interacts with part of the vinca

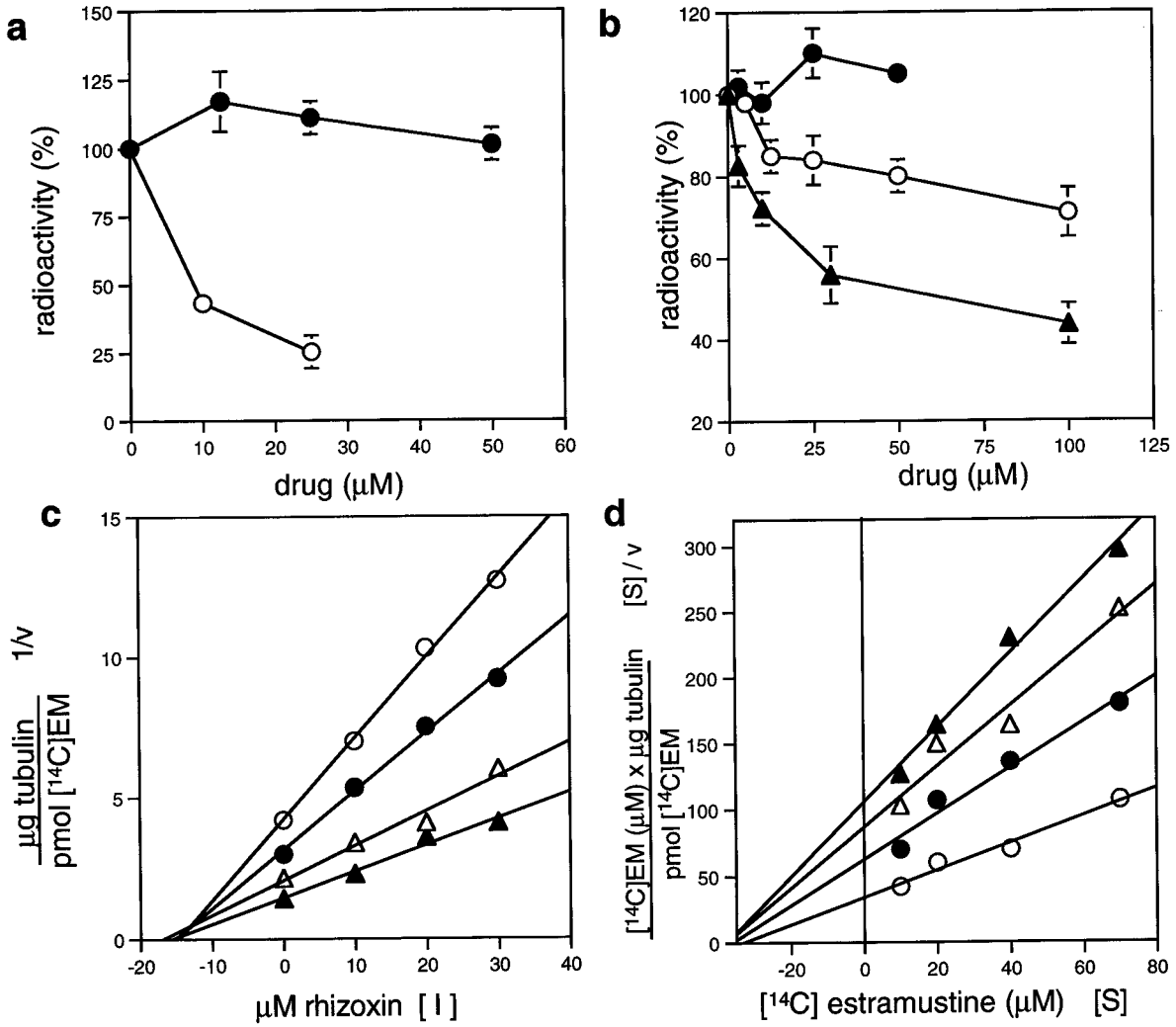

FIGURE 5: Interaction of EM with the vinblastine and rhizoxin binding sites on tubulin. (a) The effect of EM (●) and unlabeled vinblastine (○) on the binding of [^{3}H]vinblastine to tubulin as detected with a gel filtration assay. (b) Quantitation of the effects of vinblastine (●), vincristine (○), and rhizoxin (▲) on the labeling of tubulin with [^{14}C]EM. Experiments were performed as described in Figure 1a with various concentrations of the three drugs. The radioactivity associated with the tubulin bands was quantitated as follows. The samples were run on 8% acrylamide gels, dired, and exposed to an imaging screen for 48 h, and the radioactivity associated with the tubulin bands was quantitated. (c) Dixon plot for the inhibition of the binding of [^{14}C]EM to tubulin by rhizoxin. The following concentrations of [^{14}C]EM were used: (○) 10 μM, (●) 20 μM, (△) 40 μM, and (▲) 70 μM. The binding of [^{14}C]EM to tubulin was quantitated by centrifugal gel filtration as described in Experimental Procedures. (d) Hanes analysis of the binding of [^{14}C]EM to tubulin in the presence of rhizoxin. The following concentrations of rhizoxin were used: (○) 0 μM, (●) 7 μM, (△) 15 μM, and (▲) 30 μM. Binding was quantitated by centrifugal gel filtration as described in Experimental Procedures.

alkaloid domain since rhizoxin and, to a lesser extent, vincristine reduce the photolabeling of tubulin with [^{14}C]-EM. Therefore, the EM binding site appears to overlap with at least part of the rhizoxin binding site of the vinca alkaloid domain on tubulin.

Paclitaxel is a novel diterpenoid with a unique mechanism of action toward microtubules. It binds to the tubulin polymer and enhances the assembly of microtubules in the absence of GTP (Nath et al., 1985). Recent evidence has indicated that β-tubulin is the target for paclitaxel since it is the predominant subunit labeled directly by [^{3}H]paclitaxel (Rao et al., 1992). Previous studies have indicated that the combination of paclitaxel and EM produces synergistic antitumor activity in cell culture (Speicher et al., 1992) and in preliminary studies in the clinic (Hudes et al., 1995). The present data indicate that paclitaxel and EM have distinct binding sites on tubulin. Since paclitaxel preferentially interacts with the β-subunit of tubulin to promote polymerization (Rao et al., 1992) and EM interacts with both subunits to promote depolymerization, it is difficult to propose a tubulin-based mechanism whereby a combination of the two drugs produces synergistic cytotoxicity. It has been reported that paclitaxel can induce a sustained mitotic block in the absence of an increase in microtubule polymer mass (Jordan et al., 1993). At concentrations substoichiometric to those of tubulin, the drug causes a kinetic stabilization of spindle microtubules and blocks mitosis. It would be of interest to determine whether EM and paclitaxel are synergistic in attenuating microtubule dynamics despite the fact that they have apparently opposing effects on microtubule polymerization.

The characteristics of microtubules are strongly influenced by the isotypic composition of the constituent β-tubulin subunits. Isotypically pure $\alpha\beta_{III}$-tubulin dimers were shown to have more dynamicity than $\alpha\beta_{II}$- or $\alpha\beta_{IV}$-tubulin dimers (Panda et al., 1994). Bovine brain microtubules depleted in the β_{III}-tubulin isotype exhibit increased MAP-induced (Banerjee et al., 1990) and paclitaxel-induced assembly (Lu & Luduena, 1993). These microtubules are also less susceptible to the depolymerizing effects of colchicine (Lu & Luduena, 1993). Treatment of cells with colchicine or paclitaxel results in preferential incorporation of $\alpha\beta_{II}$ dimers

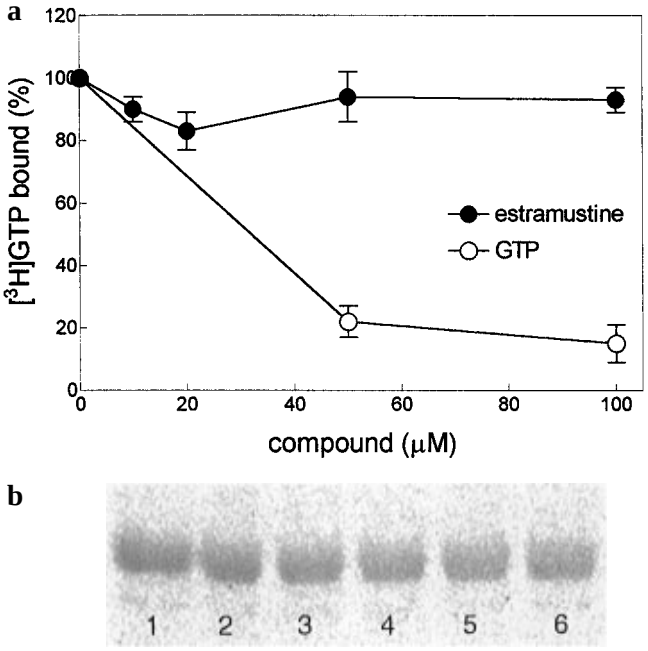

FIGURE 6: Lack of interaction between the GTP and EM binding sites on tubulin. (a) The effect of EM on the exchange of GDP for [^{3}H]GTP on tubulin. MTPs were incubated with EM or unlabeled GTP, and then [^{3}H]GTP was added. The amount of [^{3}H]GTP bound to tubulin was determined by gel filtration as described in Experimental Procedures. (b) The effect of GTP on the labeling of tubulin with [^{14}C]EM. The experiment was performed as described in Figure 1a with the following GTP concentrations: lane 1, 0 mM; lane 2, 0.1 mM; lane 3, 0.2 mM; lane 4, 0.4 mM; and lane 5, 0 mM.

(Jordan et al., 1993) and $\alpha\beta_{III}$ dimers (Laferriere & Brown, 1995) into the cellular microtubules, respectively. Therefore, treatment of cells with certain antimicrotubule agents alters the sorting of tubulin isotypes.

Recent studies from our group have shown that EM resistant human prostatic carcinoma cells have significantly higher levels of the β_{III} isotype compared to the EM sensitive cells (Ranganathan et al., 1996). In order to test the hypothesis that a possible defense mechanism against EM is an increased expression of β_{III}-tubulin, we photolabeled bovine brain microtubules with [^{14}C]EM and separated the β_{III}-tubulin isotype from α-tubulin and the rest of the β-tubulin isotypes. Separation of the β_{III} isotype by two-dimensional SDS−PAGE is based upon the change in mobility after complete alkylation of cysteine residues (Crestfield et al., 1963). In these experiments, [^{14}C]EM labeling was carried out prior to the alkylation. Since covalent labeling of tubulin with [^{14}C]EM did not alter the two-dimensional separation profile of the tubulin isotypes, it can be concluded that cysteine residues were not involved in the covalent bond formation with EM upon irradiation.

The different isotypes of β-tubulin exhibit almost complete homology within mammalian species (Sullivan, 1988). Our results indicate that the EM binding characteristics of the bovine tubulin isotypes are similar to those of the human isotypes. The bovine β_{III}-tubulin isotype incorporated [^{14}C]-EM with a specific activity that was $^1/_3$ of that of the other tubulin isotypes, while significant labeling of human β_{III}-tubulin was undetectable. This would suggest that the β_{III} isotype binds EM with a much lower affinity or it has fewer binding sites per subunit than the other β-tubulin isotypes present. The amino acid sequence divergence between the different β-tubulin isotypes is 4−16%, and about half of the differences represent conserved changes (Sullivan, 1988). It is therefore unlikely that the 3-fold difference in the incorporation of [^{14}C]EM into β_{III}-tubulin versus that of the other isotypes is due to a significant difference in reactivity of the protein. The number of photoactivated [^{14}C]EM molecules in close proximity to the different proteins would then determine the relative amounts of labeling observed. According to these results, cells expressing an increased proportion of β_{III}-tubulin should be less susceptible to the

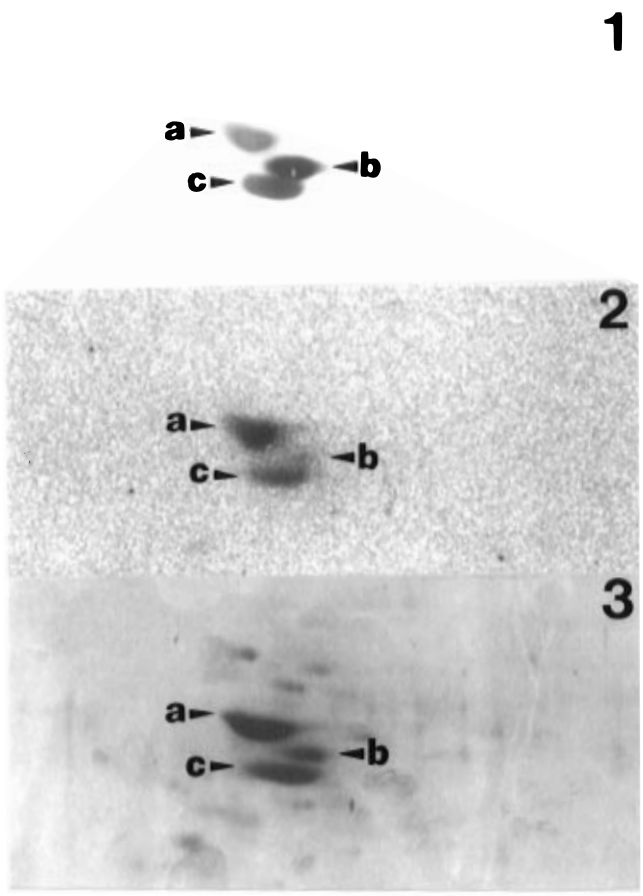

FIGURE 7: Two-dimensional SDS−PAGE of alkylated bovine microtubule proteins irradiated in the presence of [^{14}C]EM. Individual protein spots are identified as (a) α-tubulin, (b) β_{III}-tubulin, and (c) β-tubulin depleted in the β_{III} isotype. (Panel 1) Western blot developed sequentially with antibodies to α-tubulin and tubulin isotypes II−IV. The Western blot was developed after each primary antibody to ensure the position of each protein. Several blots were probed and developed with the antibodies in different orders. The tubulin isotypes β_{II} and β_{IV} had overlapping positions on the blot. (Panel 2) Radioactivity covalently associated with the microtubule proteins on the blot. (Panel 3) Silver-stained gel.

Table 1: Specific Activity of [^{14}C]EM-Labeled Tubulin Isotypes

protein	specific activity[a] [mol of [^{14}C]EM/ (molof tubulin)] of bovine brain MTPs
α-tubulin	0.055 ± 0.014
β-tubulin[b]	0.072 ± 0.020
β_{III}-tubulin	0.023 ± 0.009

[a] Bovine brain MTPs were photoaffinity labeled with [^{14}C]EM, alkylated, and then subjected to two-dimensional SDS−PAGE. [b] Covalent labeling was quantitated by exposing dried gels and blots to an imaging screen for 7 d and evaluating the level of radioactivity associated with the tubulin isotypes. The relative amounts of protein in the tubulin isotypes were determined by scanning stained gels on a laser densitometer. Specific activity was determined by dividing the units of radioactivity by the protein in relative units. The data were the average of three separate experiments ± standard deviation. [c] β-Tubulin depleted of the β_{III} isotype.

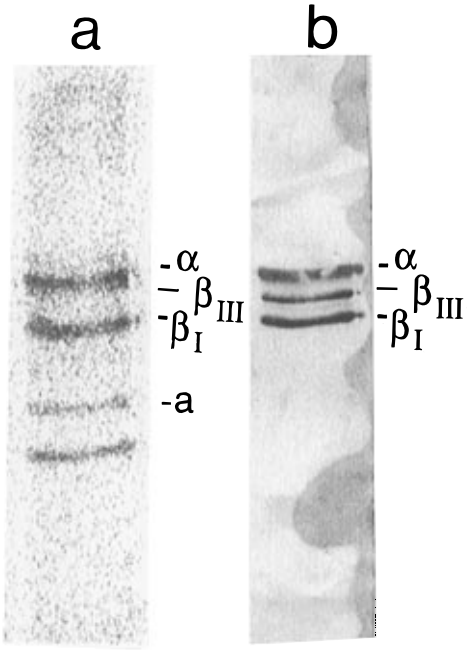

FIGURE 8: One-dimensional SDS−PAGE of cytoskeletal proteins from DU145 cells. Proteins were prepared and labeled as described in Experimental Procedures. (a) Radioactive image. The positions of α-, β_{III}-, and the other β-tubulin isotypes (β_I being the predominant isotype) are indicated. One of the two additional proteins labeled by [^{14}C]EM was identified as actin (labeled with an a) by Western blotting (data not shown). The identity of the lowest molecular weight protein labeled is unknown. (b) Western blot indicating positions of α-, β_{III}-, and β_I-tubulin. The two images were aligned according to the positions of molecular weight markers.

antimicrotubule effects of EM. The binding of colchicine to different β-tubulin isotypes has been measured (Banerjee & Luduena, 1992). The $\alpha\beta_{III}$ dimer was shown to have an affinity for colchicine that is 2-fold lower than that of $\alpha\beta_{II}$ and 28-fold lower than that of $\alpha\beta_{IV}$. The levels of β_{III}-tubulin in human tumor cells may therefore be a significant determinant of response to treatment with either colchicine or EM. Since removal of the β_{III} isotype has been shown to enhance paclitaxel-induced microtubule assembly (Lu & Luduena, 1993), it would follow that cells expressing an increased proportion of β_{III}-tubulin would be less sensitive to the effects of paclitaxel. Accordingly, we have found that our EM resistant cells exhibit 2−3-fold resistance to paclitaxel (Ranganathan et al., 1996). An increased understanding of the β-tubulin isotypic changes which bring about resistance to a specific antimicrotubule agent should help in the design of more effective combination chemotherapy employing more than one antimicrotubule drug.

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