Estramustine Depolymerizes Microtubules by Binding to Tubulin

Björn Dahllöf, Anita Billström, Fernando Cabral, and Beryl Hartley-Asp
Kabi Pharmacia Oncology, S-223 63 Lund, Sweden [B. D., A. B., B. H-A.], and Department of Pharmacology, University of Texas Medical School, Houston, Texas 77225 [F. C.]

INTRODUCTION

EM is an antineoplastic drug used in the treatment of advanced prostate cancer. The active compound consists of a 17β-estradiol group linked to nitrogen mustard via a carbamate bridge (Fig. 1). Clinically, EM is given as the prodrug EMP, which is subsequently dephosphorylated when administered both p.o. and i.v. (1).

EM was designed to function as an alkylating drug targeted to estrogen receptor-bearing cells (e.g., breast cancer cells). There, the carbamate bridge would be cleaved to release non-nitrogen mustard. However, the compound was found to be devoid of alkylating activity due to unanticipated stability of the carbamate bridge and lack of alkylating activity of the intact molecule (2, 3). Also, the intact estramustine molecule did not show any significant binding to estrogen receptors (3, 4). For a review of these properties of EM, see Ref. 5.

Although EM did not exhibit the predicted properties for which the drug was developed, the intact molecule had pronounced cytotoxicity independent of any hormonal or alkylating activities (3, 5, 6). It was shown that EM belongs to the group of antimiotic drugs, such as the Vinca alkaloids and colchicine, which can disrupt the microtubule network of cells in tissue culture and block cells at mitosis (7). Also, EM has recently been shown to block cells at mitosis in prostate tumor xenografts (8).

Experiments using purified microtubule proteins from rat brain and from DU 145 human prostate cancer cells suggested that the antimiotic effects of estramustine were caused by binding of the drug to MAPs (9, 10). Such an interaction, revealed by binding of radiolabeled EM to MAPs and by the ability of EM to strip MAPs from taxol-stabilized microtubules in vitro, was believed to lead to disassembly of microtubules. However, it should be noted that these authors also showed binding of EM to tubulin but interpreted this as being nonspecific. Investigators (11) from another laboratory also reported binding of EM to both tubulin and MAPs but could not detect any depolymerizing activity of EM in vitro. Thus, the actual target for the antimicrotubule activity of EM has not yet been unequivocally determined.

Curiously, it was found that the prodrug EMP also possessed antimicrotubule activity, but only in microtubule polymerization experiments in vitro (12, 13). In experiments using cultured cells, EMP either is inactive because it cannot penetrate the plasma membrane (see “Results”) or demonstrates activity identical with that of EM. The latter result was found in glioma cells and was shown to be due to dephosphorylation of EMP by cellular phosphatases (14). The microtubule-depolymerizing activity of EMP in vitro was shown to be due to interactions between the negatively charged estramustine phosphate molecule with the positively charged tubulin-binding domains of MAPs (15, 16). It is important to remember that this interaction is purely an in vitro phenomenon which is irrelevant for the intracellular mode of action of EM. Unfortunately, in the vast majority of the literature concerning EM, the proper distinction between the modes of action of EM and EMP has not been made.

In order to study the mechanism of action of EM, we have compared the cytotoxic and antimicrotubule effects of EM with those of VLB, a well-characterized antimiotic drug with affinity for tubulin, which recently has been combined with EM for the treatment of advanced prostate cancer (17, 18). Our results show that the two drugs are qualitatively similar with respect to cellular effects, which suggests a common mode of action. This prompted us to carry out further experiments to challenge the hypothesis that EM exerts its action via binding to MAPs. Evidence is presented which shows that EM, both in vivo (cell culture) and in vitro, causes microtubule disassembly by interaction with tubulin.

MATERIALS AND METHODS

Cell Culture. The DU 145 cell line (purchased from American Type Culture Collection) was derived from a metastatic brain lesion of a human prostate adenocarcinoma and cultured as previously described (6). Growth conditions and characteristics of the CHO cell lines have previously been described (19).

Drugs. EM (estradiol-3-N-bis(2-chloroethyl)carbamate) and EMP were synthesized by Kabi Pharmacia (Lund, Sweden). VLB was purchased from Lilly, and taxol was from Sigma or as a gift to F. C. from Dr. Matthew Suffness of the National Cancer Institute. For each experiment, fresh stock solutions were made in DMSO and diluted in medium to a final concentration of 0.1% DMSO.

Antibodies. Mouse anti-β-tubulin (N357) was purchased from Amersham; alkaline phosphatase-conjugated rabbit anti-mouse antibody (S3721) was from Promega; fluorescein isothiocyanate-conjugated goat anti-mouse antibody (F479), and tetramethylrhodamine B isothiocyanate-conjugated swine anti-rabbit antibody (R156) were from Dako A/S, Denmark. Rabbit antisera recognizing CHO-MAPs was raised against a heat-stable protein (M, 210,000).
that copurifies with microtubules from CHO cells and is believed to be the hamster MAP4 (20).

Assays for Cytotoxicity. The cytotoxic effects of EM, EMP, and VLB on survival of DU 145 cells were determined by using a colony-forming assay. Logarithmically growing cells were suspended using 85 mM sodium citrate, diluted, and seeded in 5-ml Petri dishes at a concentration of 210 cells/ml with a plating efficiency of approximately 20%; 24 h later, the dishes were randomly divided into groups of 5 prior to treatment with drug for 24 h. Thereafter, the cells were incubated for another 11 days with a change of medium at day 7, fixed in 100% methanol at -20°C, and stained with 1.0% methylene blue, and colonies composed of >50 cells were scored. Results are presented as percentages of surviving cells compared to nontreated cells. Drug resistance of CHO cells was determined by seeding approximately 100 cells into each well of a 24-well dish. Cells in replicate wells were then exposed to various concentrations of the drug for 5–7 days, and surviving colonies were stained with 0.5% methylene blue as previously described (21).

Mitotic Index. DU 145 cells were suspended as described above and diluted to 105 cells/ml. Culture flasks were inoculated with 5 ml of the cell suspension and incubated for 2 days. Thereafter, cells were exposed to drug for 26 h, and preparation for mitotic index analysis was carried out as previously described, except that colcemid was omitted (22). At each drug concentration, 4000 cells (for the control, 8000 cells) were counted. The results are given as percentages of mitotic cells in treated cultures divided by the percentages of mitotic cells in the control.

Determination of Depolymerization in Vivo. DU 145 cells were grown to confluency in 12-well tissue culture plates, and duplicate wells were treated with drug for 3 h. Thereafter, cells were rinsed briefly in PBS without Ca2+ and Mg2+ and lysed in 300 μl of microtubule-stabilizing buffer containing taxol (20 mM Tris-HCl, pH 6.8-7.0, 140 mM NaCl, 2 mM ethylene glycol bis[β-aminoethyl ether] N,N,N',N'-tetraacetic acid, 1 mM MgCl2, 0.5% Nonilid P-40, 0.5 mM taxol) as described earlier (19). The lysates were transferred to tubes, and the wells were rinsed once with 200 μl of the same buffer and pooled with the previous solution. Tubes were spun for 10 min in an Eppendorf centrifuge at 14,000 rpm at 4°C, and the supernatants, containing the unpolymerized tubulin, were transferred to separate tubes. Residual cytoskeletal material in each well was dissolved in 480 μl of hot SDS-PAGE sample buffer (4% SDS-30% sucrose-10 mM diithiothreitol-100 mM Tris-HCl, pH 6.8-0.01% bromophenol blue) and then combined with cytoskeletal pellets suspended in 20 μl of water. To verify that the method resulted in identical amounts of protein in corresponding fractions from different wells, the cells were labeled with [35S]methionine prior to treatment with drug, and protein in each fraction was quantitated by liquid scintillation counting (data not shown).

Protein Electrophoresis and Immunodetection. The cytoskeletal fractions were taken through two rounds of freezing and thawing in order to reduce the viscosity caused by high molecular weight DNA. An equal volume of both fractions were mixed with the same volume of SDS-PAGE sample buffer and heated to 95°C for 5 min. The samples were alkylated by the addition of a 0.1 volume of 6 M iodoacetamide and incubated for 10 min at room temperature. SDS-PAGE was run in a Protean xi cell (length, 20 cm; Bio-Rad) using a standard buffer system and 14% homogeneous gels, T = 0.37%, at 10 mA of constant current overnight without cooling. Each lane contained 40 μl of sample. Rainbow molecular weight markers from Amersham (United Kingdom) were used for determination of molecular weights. The gels were blotted onto nitrocellulose filters using a semidy blotting device (Semi-dry electrophoresis; Aancos, Denmark) in 39 mM glycine, 48 mM Trizma-base, and 20% methanol for 75 min at room temperature and 100 mA. The wet filters were blocked in 5% fat-free dry milk in TBST for 30 min, incubated 30 for 30 min with the anti-tubulin antibody diluted 1:500 in TBST, washed 3 times for 10 min in TBST, incubated with alkaline phosphatase-conjugated secondary antibody diluted in TBST for 30 min, washed 3 times for 10 min in TBST, and finally developed in 100 mM Tris-HCl, pH 9.5-100 mM NaCl-5 mM MgCl2, using the color reagents nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Promega as described by the manufacturer.

Indirect Immunofluorescence. Cells were grown on 8-well plastic chamber slides (Permanox; Nunc, Denmark) for 2 days. After drug treatment, the cells were stained with 0.5% methanol at -20°C, followed by 5 min in 100% acetone at -20°C. The remaining steps were carried out at room temperature. Following fixation, the cells were washed for 10 s in PBS and 5 min in PBS/BSA (PBS with 0.2% BSA). Incubation with the primary antibodies (1:250 dilution) was carried out for 30 min and was followed by 3 washes for 10 min in PBS/BSA. Incubation with secondary antibodies (fluorescein isothiocyanate- and/or tetramethylrhodamine B isothiocyanate-conjugated antibodies diluted 1:20) was carried out for 30 min and was followed by 3 washes for 10 min in PBS/BSA. Coverslips were mounted using 1 mg/ml of p-phenylenediamine in PBS with 80% glycerol.

Purification of Bovine Brain Tubulin. Microtubule proteins were prepared from fresh bovine brain cortex in the absence of glycerol by two cycles of polymerization-depolymerization in polymerization buffer (100 mM Pipes-1 mM GTP-0.5 mM MgSO4-1 mM ethylene glycol bis[β-aminoethyl ether]-

Table 1. Effect of VLB and EM on the mitotic index of human prostate cancer DU 145 cells

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mitotic indexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM VLB</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>4 μM VLB</td>
<td>9.2 ± 1.3</td>
</tr>
<tr>
<td>4 μM EM</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>16 μM EM</td>
<td>9.8 ± 1.2</td>
</tr>
</tbody>
</table>

a Percentage of mitotic cells in treated cultures divided by percentage of mitotic cells in nontreated controls after 26 h of treatment (mean of three experiments ± SEM).

![Fig. 1. Chemical structure of estramustine and estramustine phosphate. Nor-nitrogen mustard has been linked via a carbamate bridge to estradiol (see "Introduction").](image_url)

![Fig. 2. Cytotoxicity of EM (T) and VLB (O) on DU 145 human prostate cancer cells measured as the number of clones with >50 cells 11 days after a 24-h exposure to drug. Points (bars), means (±SEM) of three experiments give IC50 values of approximately 3 nM for VLB and 16 μM for EM.](image_url)

![Fig. 3. Depolymerization of microtubules in DU 145 cells by EM and VLB. Cells were treated with drug for 3 h, and the cytoskeletal pellets (lanes P) were separated from cytosolic supernatants (lanes S), followed by SDS-PAGE, Western blotting, and immunodetection of β-tubulin (see "Materials and Methods"). For each concentration, the staining intensity in lane P should be compared to that in lane S. In the concentration range of 4-64 μM EM and 1-16 μM VLB, dose-dependent depolymerization of microtubules can be seen, which is correlated to the cytotoxicities of the two drugs (see Fig. 2).](image_url)
MODE OF ACTION OF ESTRAMUSTINE

(N,N,N',N'-tetraacetic acid, pH 6.8) (23). Tubulin was separated from MAPs by ion-exchange chromatography on MgSO₄-pretreated phosphocellulose in 20 mM Pipes-0.5 mM MgSO₄ at pH 6.8 (24). Tubulin was eluted in the void volume, and the buffer concentration was adjusted to 100 mM Pipes-1 mM GTP-0.5 mM MgSO₄, pH 6.8.

Assembly of Microtubules. Polymerization of microtubule proteins (2 mg/ml) in vitro was performed in polymerization buffer containing 10% glycerol. The glycerol had to be added in order to diminish EM binding to the test tubes and pipettes. To further minimize adsorption, stock solutions and dilutions of EM were made in glass test tubes using glass pipettes. The reaction was initiated by increasing the temperature to 37°C and was monitored by apparent change in A₃₅₀. Tested drugs were dissolved in DMSO at 1000-fold the final concentration. Thus, the final DMSO concentration was 0.1% in all experiments (including control). Phosphocellulose-purified tubulin (2 mg/ml) either (a) was polymerized in the same way except that the polymerization buffer contained an additional 8% DMSO (12) or (b) was polymerized using a suboptimal system containing 0.8 m sodium glutamate (pH 6.6) at 30°C in the absence of Mg²⁺ (25, 26).

RESULTS

Cytotoxicity and Mitotic Index. A clonogenic assay (DU 145) was used to compare the cytotoxicities of EM, EMP, and VLB. EMP caused no cell death, and high-performance liquid chromatography analysis revealed that no dephosphorylation to EM, the active compound, had occurred (data not shown). EM had an IC₅₀ value of approximately 16 µM, while the IC₅₀ for VLB was approximately 3 nM (Fig. 2). As would be predicted for antimitotic drugs, both VLB and EM caused mitotic arrest at concentrations that produced cytotoxicity (Table 1).

Depolymerization of Microtubules. At drug concentrations which represented the different parts of the cytotoxicity curves of EM...
MODE OF ACTION OF ESTRAMUSTINE

Fig. 5. Depolymerization of microtubules in DU 145 cells is reversible. Treatment was for 1 h using 64 μM EM (A, C, and E) or 16 nM VLB (B, D, and F) followed by indirect immunofluorescence at 0 (A and B), 10 (C and D), or 60 min (E and F) after washout of drug. As can be seen in E and F, microtubule networks were reformed within 1 h for both drugs. In A, bar = 10 μm.

and VLB, cells were harvested and the cytoskeletons were separated from soluble components using a taxol-containing buffer (19). The fractions were subjected to SDS-PAGE analysis followed by Western blotting and immunodetection of β-tubulin to determine the relative ratio of polymerized tubulin [Fig. 3, lanes P (pellet)] versus unpolymerized free tubulin dimers [lanes S (supernatant)]. In the control samples (Fig. 3, lanes C), the supernatant fraction shows apparently slightly higher staining intensity than the pellet fraction. For cells treated with 4, 16, and 64 μM EM, a comparison of each lane P with the corresponding lane S indicates a dose-dependent depolymerization of the microtubule network. The same result was obtained using 1, 4, and 16 nM VLB. Although exact measurements of the degree of depolymerization at each drug concentration have not been made (because of technical difficulties), we conclude that both EM and VLB cause microtubule depolymerization at cytotoxic concentrations. This indicates a similar mode of action.

Indirect Immunofluorescence. In a previous investigation (27), it was found that EM and VLB caused qualitatively different patterns of microtubules as revealed by immunocytochemistry. However, it should be noted that concentrations of EM and VLB were compared which were equipotent in inhibiting the invasion of DU 145 cells into chick heart spheroids rather than equitoxic. Using indirect immunofluorescence, we studied the microtubule networks of DU 145 cells at the same concentrations as those used for determination of degree of microtubule depolymerization. Again, we found similar dose-dependent effects on the microtubules (Fig. 4). At 4 μM EM and 1 nM VLB, the most peripheral microtubules seemed to be retracted, and the central networks showed a slightly “criss-crossed” pattern. At 16 μM EM and 4 nM VLB, most microtubules were concentrated in the perinuclear regions, and at 64 μM EM and 16 nM VLB, the microtubule networks were apparently completely depolymerized. Blebs in the plasma membranes filled with tubulin were frequently found which is a common effect of antimicrotubule drugs. All immunofluorescence experiments were analyzed by two investigators without knowledge of the conditions involved, and we repeatedly failed to determine whether cells had been treated with EM or VLB. Thus, in
DU 145 cells, EM and VLB affected the microtubule network in a qualitatively similar manner that correlated to the degree of microtubule depolymerization and cytotoxicity.

Time-course experiments using 64 μM EM and 16 nM VLB revealed that the microtubule networks were affected as early as 5–10 min after treatment. Maximal effects were seen at these concentrations after 1 h of treatment with both drugs (data not shown).

**Reversible Effects.** The modes of action of EM and VLB were further compared by studying the reversibility of microtubule depolymerization. DU 145 cells were treated with 64 μM EM or 16 nM VLB for 1 h, concentrations sufficient to cause significant and apparently similar effects on the microtubule networks (Fig. 5). After washout, microtubule networks began to reform within 10 min for both drugs. After 1 h, background staining due to unpolymerized tubulin dimers was greatly reduced, and microtubules extended to the cell periphery. Thus, neither EM nor VLB caused irreversible depolymerization of microtubules at the concentrations used.

**Mutations in Tubulin Genes Result in Resistance to EM.** Several CHO cell mutants have been isolated with mutations in tubulin genes and an altered sensitivity to microtubule drugs (19, 21, 28). For example, cells with a mutation resulting in more stable microtubules are less sensitive to depolymerizing drugs such as colcemid and VLB but more sensitive to taxol compared to wild-type cells. In contrast, mutants with less stable microtubules are more sensitive to depolymerizing drugs and less sensitive to taxol. For a further discussion of the implication and utility of these mutants, see the report by Cabral and Barlow (28).

In this study, we used mutant cells to assay whether mutations in tubulin genes could also affect sensitivity to EM. Wild-type cells, Cmd4, a colcemid-resistant mutant, and Tax5–6, a taxol-resistant mutant, were tested for their sensitivities to colcemid and EM. The results showed that the three cell lines responded to challenge to EM in a manner similar to their response to colcemid, a microtubule-depolymerizing drug (Fig. 6). Since mutations in tubulin genes affect sensitivity to EM, we conclude that the dominant contribution to the cytotoxicity of EM is its microtubule-depolymerizing activity.

**EM Treatment of Taxol-stabilized Microtubules in Vivo.** The results presented thus far indicate that EM, although being less potent, does not differ significantly from other microtubule-depolymerizing drugs in its effect on cells in culture. This outcome is compatible with either a tubulin-binding activity, similar to other microtubule drugs, or with binding to MAPs. For the latter possibility, the assumption has to be made that breaking MAP-tubulin interactions in vivo results in depolymerization of the microtubule network (the physiological role of the different MAP proteins is not yet fully understood). Previous experiments from others showed that EM had affinity for MAPs in vitro and that EM could dissociate MAPs from microtubules stabilized with taxol in vitro and in vivo (9, 10). Therefore, using a similar approach, we tested whether EM could affect the interaction between MAPs and tubulin in vivo in CHO cells.

CHO cells were treated for 1 h with 1 μM taxol to stabilize the microtubule network and then with taxol plus 64 μM EM for another 3 h. The cells were fixed and stained for indirect immunofluorescence using antibodies to both tubulin and an M, 210,000 heat-stable CHO MAP. The results were compared to those from untreated cells and from cells treated with either taxol or EM alone for the same time (Fig. 7). Occasionally, significantly larger cells were found (Fig. 7, A, B, E, and F), and these were selected for photography, when possible, because of their extensive microtubule networks. However, the results of the experiment were independent of the cell size.

In control cells, the MAP antibody decorated all the microtubules recognized by the tubulin antibody. When cells were treated with 64 μM EM, significant depolymerization of the microtubule network was seen. However, some residual microtubules persisted, and these appeared to be preferentially stained by the MAP antiserum.

Prestabilization of cellular microtubules by taxol permitted us to ask whether EM, at concentrations sufficient to cause depolymerization in the absence of taxol, would change the MAP decoration of microtubules. As can be seen in the two lower panels in Fig. 7 (compare F and G with H), the addition of EM to taxol-stabilized microtubules did not release MAPs from the microtubule network. This indicates that EM does not cause microtubule depolymerization in vivo by binding to MAPs. Thus, our results contrast with those of others regarding the effects of EM on taxol-stabilized microtubules (9, 10).

**In Vitro Polymerization of Microtubules.** To further test whether MAPs mediate the ability of EM to produce microtubule depolymerization, the effect of EM on assembly of microtubules in vitro was explored. MTP (tubulin plus MAPs) and PC-tubulin (free from MAPs) were purified from bovine brain by standard procedures (see “Materials and Methods”). The purity of each fraction was verified by SDS-PAGE analysis. With this method, MAPs were not detected in the PC-tubulin preparation (Fig. 8A, lane b).

We first tried to reproduce the results of others (9, 10) i.e., EM was found to detach MAPs from in vitro polymerized microtubules stabilized with taxol. However, SDS-PAGE analysis of precipitated microtubule pellets showed no such effects on the MAP content using similar conditions (data not shown). In the absence of taxol, EM could partially inhibit microtubule polymerization in a dose-dependent manner (data not shown). Since the latter result could be due to binding of EM to tubulin or MAPs, next we explored the effects of EM on polymerization of PC-tubulin using similar conditions for polymerization except that 8% DMSO was added to promote assembly in the absence of MAPs (12). Importantly, we found that EM could partially
MODE OF ACTION OF ESTRAMUSTINE

Fig. 7. Double-labeling indirect immunofluorescence of CHO cells using antibodies to tubulin (A, C, E, and G) or an M, 210,000 CHO-MAP (B, D, F, and H). In control cells, the MAP antibody decorates all microtubules (A and B). Treatment with 64 μM EM for 3 h causes microtubule disassembly as revealed by tubulin staining (C). However, some microtubules remain and are preferentially stained by the MAP antibody (D); see "Results" for an explanation. In E, F, G, and H, cells were treated with 1 μM taxol for 1 h to stabilize the microtubule network, followed by 3 h in the absence (E and F) or presence (G and H) of 64 μM EM. A comparison of E and F with G and H shows that EM does not dissociate MAPs from taxol-stabilized microtubules in vivo. In A, bar = 10 μm.

inhibit the polymerization of PC-tubulin in a dose-dependent manner (Fig. 8B). Using these conditions, we could not inhibit microtubule assembly to a higher degree than that shown in Fig. 8, because EM precipitates at concentrations >40 μM. Although the extent of inhibition appears to be less than that which can be achieved in vivo, it is a common observation that antimitotic drugs have less efficacy in vitro than in vivo. For example, we obtained approximately a 50% reduction in the extent of polymerization in vitro using 1 μM VLB and 5 μM colcemid (data not shown), whereas the concentration needed to reduce polymerization about 50% in vivo was approximately 4 nM (Figs. 3 and 4) and 64 nM (not shown), respectively. Thus, compared to the relation between the in vitro and in vivo effects of VLB and colcemid, the in vitro effects of EM are actually better than what may have been expected based on its in vivo effects.
MODE OF ACTION OF ESTRAMUSTINE

Fig. 8. Purification and polymerization of microtubules in vitro. MTP and PC-tubulin were prepared from bovine brains as described in "Materials and Methods." A. Coomassie Brilliant Blue-stained SDS polyacrylamide gel of (a) MTP, (b) PC-tubulin, and (c) MAPs. The identities of the bands are tentatively indicated. No staining of high M₆ MAPs can be found in the PC-tubulin fraction. Detection of tau in lanes a and b is obscured by the high amounts of tubulin in the same region of the gel. B, polymerization of PC-tubulin (2 mg/ml) in 10 and 40 μM EM compared to control. C, polymerization of PC-tubulin at suboptimal conditions in 1, 2, and 5 μM EM compared to the control. In this system, 5 μM EM results in a similar relative degree of inhibition compared to the control as 40 μM EM did in the standard assay (B). D, PC-tubulin incubated at suboptimal conditions in the presence of 40 μM EM compared to the control. E, the material in D incubated for another 15 min on ice and the absorbance measured at 5, 10, and 15 min following start of cold treatment. Thus, the structures induced by high concentrations of EM are qualitatively different compared to the control sample.

By altering the temperature, buffer composition, and concentration of Mg²⁺, others have shown increased activity of weak tubulin binders in such a suboptimal system compared to the activity in the standard polymerization assay (25, 26). Therefore, we polymerized PC-tubulin in a similar manner and found dose-dependent inhibition of microtubule polymerization at considerably lower EM concentrations (1–5 μM EM; Fig. 8C). However, at higher EM concentrations (10–40 μM), the microtubule polymerization (as detected by an increase of apparent absorbance) was not totally abolished. Rather, the shapes of the polymerization curves were gradually changed. This effect was most pronounced at 40 μM EM (Fig. 8D). Several reports have previously shown that estrogenic substances, which inhibit binding of radiolabeled colchicine to tubulin, can induce formation of cold-stable tubulin structures (for a review, see Ref. 29). Therefore, we examined the cold stability of control microtubules and the material obtained using 40 μM EM. As can be seen in Fig. 8E, control microtubules were completely depolymerized already following 5 min at 4°C, whereas a substantial absorbance was recorded for the sample containing EM even after 15 min of cold treatment. Thus, we conclude that EM can efficiently inhibit polymerization of pure tubulin in the absence of MAPs and that high concentrations, using suboptimal polymerization conditions, induce the formation of cold-stable structures similarly to what is found using other synthetic estrogens.

DISCUSSION

In this paper we compared the cellular effects of EM with those of VLB and described the following results: (a) both drugs induced mitotic arrest at their respective IC₅₀ concentrations; (b) at comparable cytotoxic concentrations, both drugs caused indistinguishable and reversible depolymerization of microtubules, as detected by cytoskeleton extraction and indirect immunofluorescence; (c) a panel of CHO cell lines with tubulin mutations exhibited similar patterns of resistance or hypersensitivity to EM and to colcemid, a well-characterized microtubule inhibitor that binds to tubulin; (d) EM was unable to displace MAPs from taxol-stabilized microtubules in vitro and in vivo; and (e) EM was shown to inhibit in vitro polymerization of purified tubulin in the absence of MAPs.

For several years, EM has been known in the microtubule field for its unique mode of action. While all other microtubule drugs interact with sites on tubulin molecules, EM was thought to bind to the MAPs and thereby disrupt microtubule assembly (9, 10, 30). In this context,
it is important to remember the different properties of EM, which is the intracellular active metabolite, and the prodrug EMP. For EMP, work from another laboratory clearly showed that high micromolar EM, like VLB, acts by an interaction with tubulin. Those of VLB. We conclude that both drugs cause mitotic arrest and to the colchicine site (39). Binding studies are now under way in order to the consequences of depolymerization of EM has indicated similarities to other drugs known to bind 11. Information of EM has indicated similarities to other drugs known to bind to the colchicine site (39). Binding studies are now under way in order to determine more precisely the nature of the EM-tubulin interaction. In a recent review, it was suggested that EM would preferentially affect the mitotic spindle (30). We found that EM and VLB affect both interphase and spindle microtubules (measured as mitotic arrest) in the same concentration range. Of course, the consequences of depolymerization of the spindle microtubules are much more devastating for the dividing cell than depolymerization of interphase microtubules, but this is true for all microtubule drugs. Recently, clinical trials have indicated that a combination of EM and VLB is beneficial for patients with advanced prostate cancer (17, 18). Part of the rationale for combining these two drugs was their distinct molecular targets. The other reason for this combination was their different toxicity profiles. Based on similar arguments, it was also suggested that the combination of taxol and EM would be a logical combination therapy approach (30). Since our review shows that EM is a tubulin-binding drug, it does not, based on mechanistic arguments, support the combinations of EM with other microtubule drugs. However, the combination of EM with drugs such as VLB may still be clinically beneficial. This may be the result of additive anti-microtubule effects without similar enhancement of the side effects. The dose-limiting toxicity of VLB is bone marrow depression, whereas cardiovascular effects are the dose-limiting complications of EM. Thus, higher concentrations of drugs with antimitotic properties, resulting in better response rates, may be obtained by giving them in combination rather than by giving a single drug.

In conclusion, we have investigated the mechanism of action of EM and compared its effect on human prostate cancer cells in culture with those of VLB. We conclude that both drugs cause mitotic arrest and microtubule depolymerization at cytotoxic concentrations and that EM, like VLB, acts by an interaction with tubulin.

ACKNOWLEDGMENTS

We would like to thank Christina Ekström, Anette Gunnessson, Ingrid Nordh, and Mats Akesson for excellent technical assistance. Furthermore, we are grateful to Dr. Margareta Wallin and her collaborators for introducing us to preparing bovine brain tubulin and to Robert Kristoffersen and Jan-Otto Holm for their darkroom expertise. Lennart Persson at SKANEK (Käfinge) is acknowledged for providing bovine brains. We wish to thank Dr. Matthew Suffness of the National Institutes of Health for the generous gift of taxol.

REFERENCES


Estramustine Depolymerizes Microtubules by Binding to Tubulin

Björn Dahllöf, Anita Billström, Fernando Cabral, et al.