Biochemical Pharmacology, Vol. 55, pp. 325–331, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00472-3

Association of Estramustine Resistance in Human Prostatic Carcinoma Cells with Modified Patterns of Tubulin Expression

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ABSTRACT. Estramustine (EM) is an antimicrotubule drug used in the treatment of hormone refractory advanced prostate cancer. To investigate the mechanism of resistance to EM, we compared its effects on human prostate cancer cells (DU145) and an estramustine-resistant derived cell line (E4). Immunofluorescence demonstrated that EM caused depolymerization of microtubules and blocked cells in mitosis in DU145 cells, with less effect in E4 cells. Using tubulin isotype-specific antibodies, a threefold increase in βIII and ~twofold increase in βI + II isotype in E4 cells compared to DU145 cells were observed. A most interesting observation concerned an increase in the posttranslational modification of α-tubulin of both polyglutamylation and acetylation in the E4 cells. Significant to this observation, using direct EM photoaffinity labeling of tubulin, drug binding to the most acidic posttranslationally modified forms of α-tubulin was shown to be minimal. Taken together, these results indicate that the modification of the tubulin expression pattern may be responsible for estramustine resistance by both lowering the amount of drug bound to microtubules and inducing more stable microtubules. BIOCHEM PHARMACOL **55**;3:325–331, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. prostatic cell lines; estramustine; drug resistance; tubulin isotype expression

Metastatic prostate cancer that is refractory to hormone therapy remains an incurable disease. Microtubules, the cytoskeletal networks that contribute to cell motility and division, have long been targets for anticancer drugs. Recently, both preclinical and clinical successes have been achieved by using combinations of antimicrotubule agents [1, 2]. Estramustine was synthesized in the early 1960s and consists of a estradiol linked to nor-nitrogen mustard via a carbamate bridge (Fig. 1). Clinically, EM¶ is given as estramustine phosphate, which is subsequently dephosphorylated when administered either p.o. or i.v. [3]. Despite its chemical structure, EM has been shown to induce mitotic arrest and microtubule depolymerization following binding to microtubule proteins [4–6]. EM demonstrates micromolar affinity for both microtubule-associated proteins (MAPs) as well as tubulin, and it remains possible that its cytotoxic properties are a composite function of these drug properties [7, 8]. Using direct photoaffinity analog techniques, we have previously shown that both α-and β-tubulin subunits are targeted by EM with affinity constants of approximately 20 μM. The EM binding site was distinct from that of colchicine and vinblastine with partial overlap with that of taxol [9].

The ability of tumors to develop acquired resistance to drugs used for treatment remains a major obstacle to successful chemotherapy. In this study, we used a human prostatic carcinoma cell line (DU145) and an estramustine resistant cell line (E4). E4 was established by selecting the DU145 cell line with stepwise increments of EM and this cell line has characteristics distinct from the standard MDR phenotype [10]. However, we have not previously analyzed this resistant cell line with respect to β-tubulin isotype expression or post-translational modifications of tubulin.

Tubulin, the major protein of microtubules, is composed of two 50-kDa polypeptide subunits, α and β, which display a high level of polymorphism. In higher eukaryotes, this diversity is due to the expression of six isogenes encoding for each α or β subunit as well as a large number of different posttranslational modifications [11–13]. MAPs are a group of molecules that were first defined on the basis of their ability to bind to and promote the assembly of tubulin. MAP expression in cells is also characterized by heterogeneity. Despite considerable effort, the functional significance of the tubulin and/or MAP isotypes in cells remains

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¶ *Abbreviations:* EM, estramustine; MAP, microtubule-associated protein; MDR, multidrug resistance; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid.

Received 28 March 1997; accepted 30 July 1997.

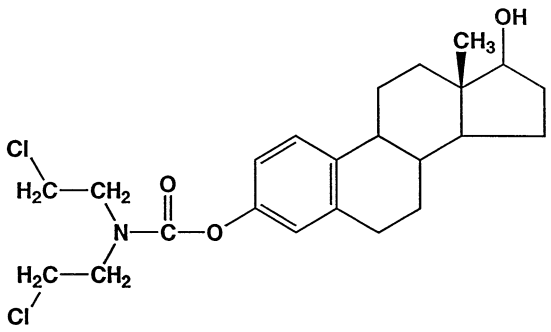


FIG. 1. Chemical structure of estramustine. Nor-nitrogen mustard is linked to estradiol through a carbamate-ester bond.

obscure [14]. Although it is unclear how microtubule functions are controlled in cells, recent evidence indicates that the dynamic properties of microtubules may play an important role. Further contributing to the properties of microtubule polymerization dynamics is the potential for tissue and/or cell types to qualitatively and quantitatively express distinct tubulin isoforms. To identify the mechanism of drug resistance, we have investigated the heterogeneity of tubulin isoforms in DU145 and E4 cells.

MATERIALS AND METHODS

Cell Culture

Human prostatic carcinoma cell line (DU145) and the estramustine-resistant cell line (E4) were developed as previously described [10]. Cells were cultured in complete media (Dulbecco's Modified Eagle's Media supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% fetal bovine serum) and incubated at 37° in a humidified 5% CO_2 atmosphere.

Drugs

EM (estradiol-3-*N*-bis(2-chloroethyl)carbamate) solutions were prepared in ethanol and frozen until use. Taxol was purchased from Sigma. [^{14}C]EM (2.5 mCi/mmol) was obtained from Pharmacia.

Cytotoxicity Assays

A total of 2.5×10^3 cells were plated in 96-well microtiter culture plates for 96 hr at 37°. The quantification of surviving cells in the presence of increasing concentrations of EM was determined by measurement of the endogenous enzyme hexosaminidase [15] and compared to untreated cells. Experiments were performed in triplicate. IC_{50} is the concentration inhibiting 50% cell growth.

Immunofluorescence and Confocal Scanning

The cultured cells grown in monolayer in 8-well Lab-tek chamber slides were treated with EM for 1 hr. Following drug treatment, cells were fixed in cold methanol. Incubation with the primary antibody was carried out overnight at 4°. Cells were then washed several times with PBS and incubated with FITC-labeled goat anti-mouse IgG (Amersham) for 45 min at room temperature. Following several rinses with PBS, coverslips were mounted using glycerol (Dako). Fluorescence images were photographed using an MRC-600 (BioRad) confocal microscope.

Protein Electrophoresis and Immunodetection

Samples were prepared by lysing exponentially growing cells in 1% sodium deoxycholate in the presence of protease inhibitors. Lysates were sonicated on ice and centrifuged at $100{,}000 \times g$ for 30 min. The supernatant was removed and protein content determined by BCA protein assay (Pierce). Equivalent amounts of protein from each sample were electrophoresed on 8% gradient SDS-PAGE. Rainbow molecular weight markers from Amersham were used for determination of molecular weights. Proteins were transferred onto nitrocellulose and Western blots were done using an antitubulin antibody, followed by a secondary antimouse IgG alkaline phosphatase conjugate and visualized by the color reagents nitroblue tetrazolium and 5-bromo-4-chloro-3 indoyl phosphate (Sigma, as described by the manufacture). Six monoclonal antibodies directed against tubulin were used in this study. The monoclonal anti-polyglutamylated tubulin GT335 was prepared in our laboratory [16]. General anti-α-tubulin and β-tubulin, anti-βIII-tubulin isotype anti-βI + II-tubulin isotypes and anti-acetylated tubulin were purchased from Sigma.

Bovine Brain Tubulin Purification

Bovine brain microtubules were prepared according to the pH- and temperature-dependent method of Tiwari and Suprenant [17]. 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 [18], to separate tubulin from the MAPs.

Photoaffinity Labeling of Bovine Tubulin with [^{14}C]EM

For direct labeling with [^{14}C]EM, tubulin (5 μM in tubulin in 0.1 M MES, 0.5 mM $MgC1_2$ and 5% DMSO at pH 7.0) was incubated with 10 μM [^{14}C]EM for 20 min at 22° and the proteins then irradiated for 10 min in a Stratagene UV linker with a 254 nm filter (3000 μwatts/cm^2).

Two-Dimensional SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell lysates were made 9.5 M in urea and subjected to isoelectric focusing in cylindrical gels (18 cm long × 0.25 cm) essentially as described previously [16], except that pH 5–6 ampholytes (Serva) were used. Proteins were focused overnight at 750 V then for 4 hr at 1,500 V. Second

dimension was run in 0.1% SDS, 8% acrylamide-0.108% bisacrylamide slab gel (14 × 24 × 0.1 cm) overnight at 8 mA per gel. Proteins were then transferred onto nitrocellulose (Hybond C) as described [19].

Isoelectric focusing of irradiated protein samples was performed according to the method of O'Farrell [20] using shorter cylindrical gels (11 cm and 0.25 cm) and pH3–10 and pH4–6 ampholytes (Pharmacia). The second dimension was carried out on 8% polyacrylamide gels [21] followed by transfer of proteins to a PVDF membrane. After exposure to a [^{14}C]-sensitive imaging screen for 24 to 158 hr, the blots were then probed with an antibody to α-tubulin, and then probed with an anti-β-tubulin antibody and developed to localize the proteins. Quantitation of radioactivity associated with the two lobes of the α-tubulin spot was determined from the phosphoimage data and the relative amount of protein from the Western blot developed with α-tubulin antibody. Each was measured through the NIH image software program. The specific activity of incorporation of [^{14}C]EM into the acidic and basic isoforms of tubulin was then calculated in arbitrary units by dividing the level of radioactivity observed in each lobe by the amount of protein.

RESULTS

Dose-dependent cytotoxicity of EM was found in DU145 and E4 cells with IC_{50} values of 2.3 μM and 17 μM, respectively. E4 cells were 7-fold resistant to EM compared to DU145 cells; these results are in good agreement with those previously reported [10]. The effect of EM on microtubules in cells was observed by indirect immunofluorescence microscopy, using an anti-α-tubulin primary antibody and an FITC-labeled secondary antibody. It was shown that EM induced a dose-dependent disassembly of microtubules. Figs. 2A and 2B show the controls of DU145 and E4 cells, respectively: E4 cells are on average 20% smaller than their wild-type counterparts. The microtubule disassembly occurred from the peripheral margins towards the cell center. In DU145 cells treated with 30 μM EM for 1 hr (Fig. 2C), the most peripheral microtubules were retracted: the microtubule networks diminished *ca.* 23% in size while the overall cell size remained unchanged (data not shown). At 60 μM, most microtubules were concentrated in the perinuclear regions and the central networks were randomly oriented (Fig. 2E). The microtubule networks were apparently completely depolymerized at 120 μM (Fig. 2G). In contrast, only 20% of E4 cells were affected by EM. Microtubule networks remained organized in E4 cells treated with 120 μM EM (Fig. 2H).

Analysis of α-and β-tubulin content by immunoblotting of total cell lysates with anti-α (DM1A) and β-tubulin (DM1B) antibodies revealed a slight decrease of α-tubulin in E4 cells compared to the DU145 cells. In contrast, the β-tubulin content appeared slightly higher in E4 than in DU145 cells (Fig. 3b). Since these antibodies are expected to recognize all α- and β-tubulin isoforms, these observations could suggest that the α/β ratio is unbalanced in DU145 and/or in E4 cells, unless the antibodies used reacted differently with the tubulins expressed in each cell line. Indirect immunofluorescence microscopy with α-tubulin antibody showed an increase stain in DU145 cells compared to E4 cells, while the β-tubulin antibody showed weak staining in DU145 cells (data not shown).

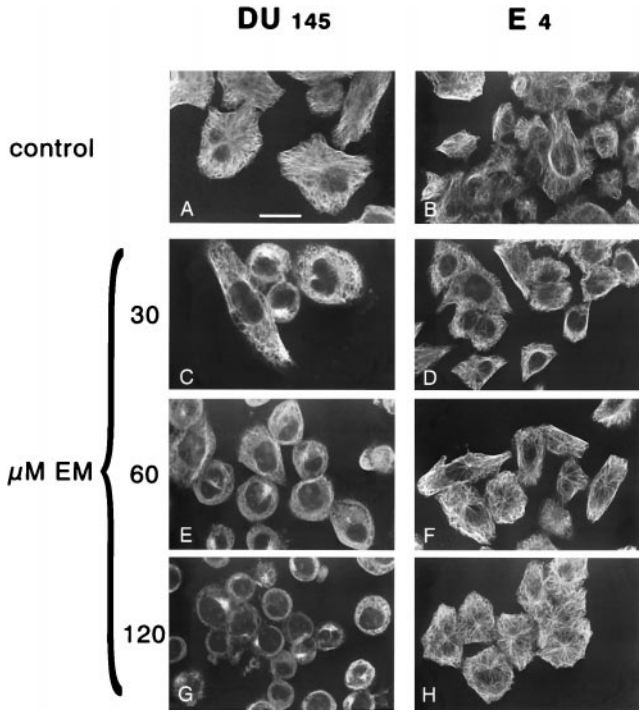
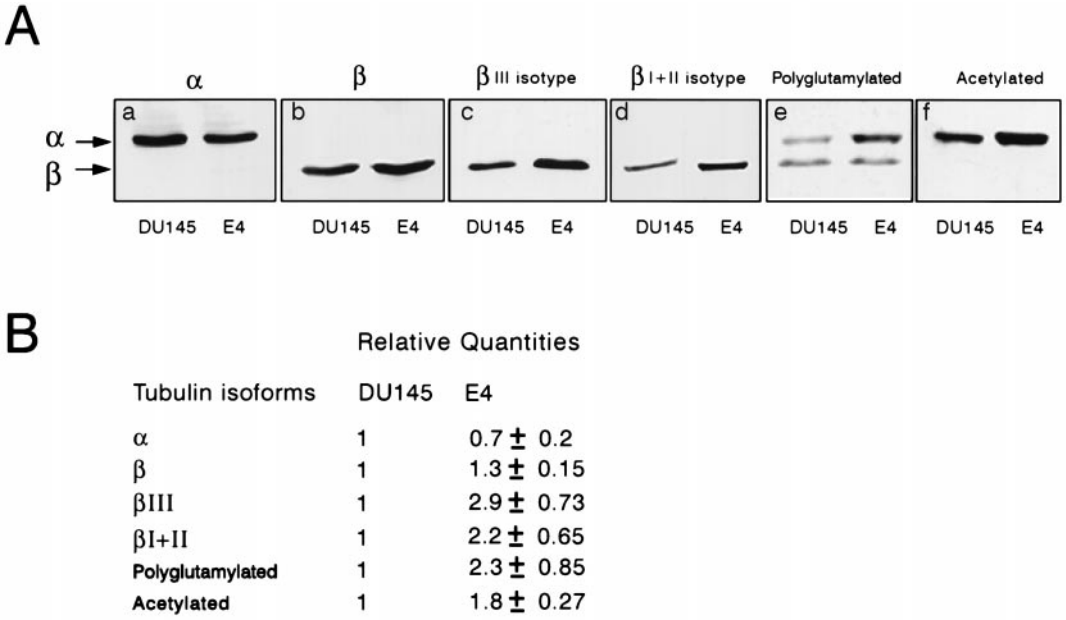


FIG. 2. Depolymerization of microtubules in DU145 and E4 cells. (A) control DU145 cells; (B) control E4 cells; (C, E, G) DU145 cells–1 hr treatment with 30, 60 and 120 μM EM, respectively. Complete disassembly was seen at 120 μM EM for DU145 cells but not in E4 cells. Bar scale = 25 μm.

To further analyze the tubulin content of these cells, antibodies directed against either distinct tubulin isotypes (primary translation products defined by their specific amino acid sequences) or specific posttranslational modifications of tubulin (acetylation, polyglutamylation) were used. Figures 3c and d show an increase in the accumulation of both βIII and βI + II isotype in E4 cells versus DU145 cells (threefold and twofold, respectively). Polyglutamylation is a posttranslational modification specific to α- and β-tubulin [22] which accumulates preferentially in neurons [16] and in axonemes [23]. It consists of the progressive addition of 1 to 6 negatively charged glutamyl units in the carboxy-terminal domain of both tubulin subunits. Figure 3e shows that polyglutamylated tubulin, as detected with the specific monoclonal antibody GT335, accumulated twofold in E4 cells, mostly at the level of the α-tubulin subunit. Similarly, acetylated α-tubulin appeared to be more abundant in E4 than in DU145 cells (Fig. 3f).

To obtain more precise information about tubulin isoforms, proteins from DU145 and E4 cell lysates were analyzed by 2-D PAGE. Figures 4a and b show an accumulation of acidic α-tubulin variants in E4 cells, likely due to



B

Tubulin isoforms	DU145	E4
	Relative Quantities	
α	1	0.7 ± 0.2
β	1	1.3 ± 0.15
βIII	1	2.9 ± 0.73
βI+II	1	2.2 ± 0.65
Polyglutamylated	1	2.3 ± 0.85
Acetylated	1	1.8 ± 0.27

FIG. 3. (A) Total tubulin and tubulin isoform content in DU145 and E4. Cell lysates from exponentially growing cells were applied to 8% polyacrylamide gels, and immunostaining was performed with α-and β-tubulin monoclonal or tubulin isotype-specific antibodies. a) α-tubulin; b) β-tubulin; c) βIII isotype; d) βI + II isotype; e) polyglutamylated tubulin; f) acetylated tubulin. (B) Relative quantities of tubulin isoforms represented in A. Each number represents an average of three independent experiments.

acetylated and glutamylated isoforms (Fig. 4b, arrow). Figures 4e and f clearly show that the level of accumulation of βIII isotype was strongly increased in E4 cells, confirming data found previously in other EM-resistant cell lines [24]. The βIII isotype tubulin is characterized by its special coordinates on 2-D gels; it migrates as a satellite species slightly more basic than the rest of β-tubulin and with a reduced mobility [25]. In these cells, it is resolved into 2 distinct isoforms, the acidic spot corresponding to a phosphorylated derivative (Figs. 4c–h). Blots revealed with the anti-βIII isotype f were re-incubated with the general DM1B antibody to allow a precise superposition and comparison of the resulting signals. The corresponding 2-D patterns (Figs. 4g and h) indicate that the polymorphism of the non βIII isotype tubulin was modified in E4 cells relative to DU145 cells: a basic isoform was no longer visible (Figs. 4g and h, thin arrow), whereas an additional acidic isoform accumulated, likely due to an enhanced posttranslational modification (Fig. 4h, thick arrow). Finally, the use of the GT335 antibody (Figs. 4i and j) led to the observation that polyglutamylation was enhanced in E4 cells, mostly at the level of α-tubulin, confirming the results shown in Fig. 3e. At the level of the β-tubulin subunit, the class III β-tubulin does not appear to be polyglutamylated in these cells. Concerning the other, non class III β-tubulin, the acidification of the β-tubulin spot observed in Fig. 4 in E4 as compared to DU145 extracts (Figs. 4h and j vs. 4g and i) appears to be due to polyglutamylation and strongly suggests that the polyglutamyl chains carried by these β-tubulins were lengthened with one additional, negatively charged glutamyl residue in E4 cells.

Altogether, these results show that the acquisition of EM resistance in E4 cells is associated with important changes in tubulin expression and accumulation. In particular, the amount of the neurospecific βIII isotype tubulin gene products is strongly increased in resistant cells, as well as the levels of the posttranslational acetylation and polyglutamylation.

Direct photoaffinity labeling of bovine brain tubulin with ^{14}C-estramustine is shown in Fig. 5. The most acidic components of α-tubulin corresponding to the posttranslationally modified forms were not covalently linked to the labeled drug. Quantitation of the specific activity of incorporation of [^{14}C]EM into the two lobes of α-tubulin on the blots showed that the more acidic forms of the protein incorporated 3-fold less [^{14}C]EM than the rest of the α-tubulin isoforms. This result would suggest that the acidic forms of α-tubulin have a lowered affinity for the drug.

DISCUSSION

In this paper, we have compared the effects of estramustine in a human prostatic cancer cell line (DU145) and in an estramustine-resistant cell line (E4). The cytotoxicity of EM has been linked to its capacity to interact with microtubules, causing cytoskeletal dysfunction and microtubule depolymerization. Our study was to evaluate, at a molecular level, the possible relationship between microtubule protein alteration and resistance to EM in E4 cells.

Our results from 1D and 2D immunoblotting using specific antitubulin antibodies revealed that tubulin was enriched in βIII and I + II isotypes and was more heavily polyglutamylated and acetylated in E4 cells than in DU145 cells. It has been shown that post-translational modifica-

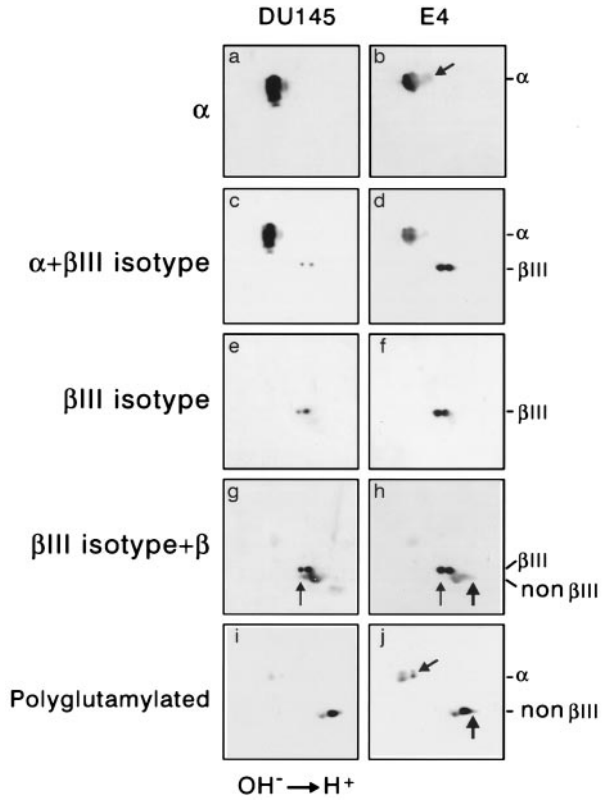


FIG. 4. Whole cell lysate proteins from DU145 cells (a, c, e, g and i) and E4 cells (b, d, f, h and j) were separated by 2-D PAGE and transferred onto nitrocellulose. Tubulin was probed with the general anti-α-tubulin monoclonal antibody DM1A (a, b), the specific anti-βIII isotype tubulin monoclonal antibody SDL 3-D10 (e, f), and the specific anti-polyglutamylated α- and β-tubulin monoclonal antibody GT335 (i, j). In some cases, blots were reincubated with a different primary antibody to allow a precise superposition of the signals: Blots a and b were reincubated with SDL 3-D10 (c and d, respectively) and blots e and f with the general anti-β-tubulin monoclonal antibody DM1B (g and h, respectively). Positions of migration of α-, β_{III}-and β-tubulin are indicated. Acidic (acetylated and glutamylated) α-tubulin isoforms are indicated by horizontal arrows, and basic and acidic isoform of non βIII by thin and thick vertical arrows.

tions of tubulin such as acetylation or detyrosylation are not the cause of microtubule stability but rather its consequence [26, 27]. Otha *et al.* [28] have reported an increased acetylation of α-tubulin in paclitaxel-resistant H69 cells. Similarly, primary cultured mouse neurons treated with taxol increase their levels of polyglutamylation and acetylation [29]. These results can be explained by the substrate specificity or preference (microtubule vs. unassembled tubulin) of the corresponding modification enzymes. Thus, an increased microtubule stability in E4 cells would logically lead to an accumulation of posttranslationally modified tubulin isoforms. Among these modifications, polyglutamylation could in turn positively influence microtubule stability, as it has been shown in neurons that this modification regulates the binding of the microtubule-associated proteins Tau and MAP2 as a function of chain length [25, 30]. The significant elongation of the polyglutamyl chains of α- and β-tubulin observed in E4 cells (Fig. 4) could increase their affinity for the MAPs and thus increase microtubule stability. These stabilized microtubules might be less sensitive to depolymerization by EM. Furthermore, the photoaffinity data suggest that EM does not bind to the most acidic posttranslationally modified forms of α-tubulin (Fig. 5). This strongly suggests that EM does not bind effectively to the acidic forms of this protein. In mammalian cells in culture, cytoplasmic microtubules that are resistant to nocodazole or colchicine contain more acetylated α-tubulin than the rest of the cellular microtubules [31]. Increased acetylation of α-tubulin and βIII-isotype has also been observed in T cell resistant to vincristine [32].

The βIII isotype is normally "specifically" expressed in neurons, where it represents a very early marker of differentiation [33]. Given the 2-D coordinates and its reactivity towards the specific antibody SDL 3-D10 (Fig. 4), there is no doubt that class III β-tubulin is effectively expressed in DU145 cells and at much higher levels in E4 cells. This result is essentially consistent with other EM-resistant cell lines where we previously found increased expression of βIII at both the protein and transcript level. In DU145 and E4 cells, βIII is resolved into 2 spots, the basic one corresponding to the primary translation product and the acidic one to its phosphorylated derivative. Whereas the total amount of βIII (basic and acidic spots) is clearly increased in E4 cells, the relative proportion of phosphorylated species is lower in E4 than DU145 cells. The altered βIII expression is consistent with our recent studies showing that EM does not bind to this isotype [9]. This would suggest that a greater proportion of the βIII isotype would allow continued microtubule function even in the presence of the drug. Presumably, there will be some intrinsic disadvantages to such βIII stoichiometry, since higher levels are only produced under selective drug pressure. The precise nature of such disadvantages are not apparent from the present data, but it would not seem unreasonable to assume that changes in structure/function may be limited to the altered dynamics of dimer composition. It is also worth noting that intracellular drug concentrations were shown to be lower in E4 cells than in wild-type cells [10]. This could be a composite function of reduced target binding and increased efflux through a non *p*-glycoprotein-mediated mechanism [34].

From these data, it is shown that the posttranslational modification and altered isotypic expression of tubulin may have an impact on the cellular sensitivity to a number of antimitotic drugs. Moreover, cells expressing "naturally" high levels of βIII isotype such as neurons (25% of total β-tubulin expression) are sensitive to the antimicrotubule drug nocodazole but do display an intrinsic resistance to EM (data not shown).

Since microtubule infrastructure is also influenced by MAP interactions with tubulin, it will be of future interest to characterize MAP expression in the resistant cells. Estramustine binding to MAPs [5, 7, 8] may also contribute to the antimicrotubule activity of the drug. It is likely that

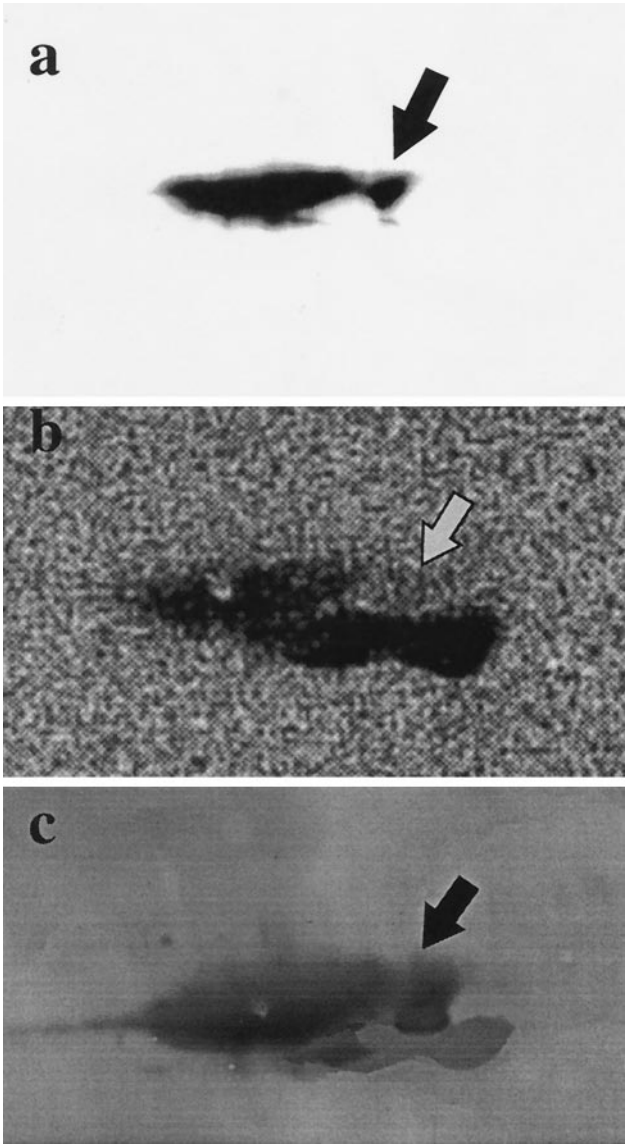


FIG. 5. Bovine brain tubulin (50 μg) was irradiated in the presence of [^{14}C]EM and subjected to 2D SDS-PAGE. The 2nd dimension was an 8% polyacrylamide gel. Fig. 5a shows the Western blot developed with an anti-α-tubulin antibody. The most acidic isoform of α-tubulin is marked with an arrow in all 3 panels, 0.55 vs. 0.19 arbitrary units of [^{14}C]EM per μg protein for the acidic isoforms vs. the rest of the α-tubulin. In Fig. 5b, the radioactive image of the blot shows where [^{14}C]EM was covalently incorporated into the proteins. Fig. 5c is a Western blot first probed with an α-tubulin, developed, and then probed with an anti-β-tubulin antibody and developed. The α-tubulin band in panel c is wider than in panel a because of the prolonged development time during the second development. These results show that the acidic isoforms of α-tubulin labeled with a significantly reduced specific activity compared to the rest of the α-tubulin.

the altered expression of tubulin isotypes, together with different patterns of post-translational modifications, will impact upon MAP-tubulin interactions.

Thus, as part of the overall pleiotropic response to drug-induced stress, acquired resistance to EM is a function of multiple adaptations. Contributing to the phenotype, altered expression of tubulin isotypes and the posttranslational modification of these proteins reduces their potential to act as drug targets.

We are grateful to Dr. J. Vassy for her assistance with confocal microscopy; Dr. A. Koulakoff for providing primary cultures of neurons; S. Lazereg for her technical assistance and Dr. L. Le Moyec for her useful suggestion for cytotoxicity experiments. This work was supported by the Saint-Louis Foundation.

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